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ROLE OF XANTHINE OXIDASE IN THE DEVELOPMENT OF SPONTANEOUSLY OXIDIZED FLAVOR IN MILK[1]

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SUMMARY

The role of xanthine oxidase in the development of spontaneously oxidized flavor was considered. The study included: (1) the relationship of xanthine oxidase activity to the development of off-flavor, (2) a multiple-enzyme system versus a single enzyme in the development of the off-flavor, and (3) the effect of inhibitors upon the development of spontaneously oxidized flavor. Results of the study indicated that: (1) The occurrence of a spontaneously oxidized flavor in milk was dependent upon a high level of xanthine oxidase activity, (2) a single enzyme (xanthine oxidase) was involved, and (3) inhibitors of xanthine oxidase were effective in preventing flavor development. A brief discussion of the peroxidatic action of xanthine oxidase was included.

In 1943, Roberts (19) reported significant correlations between the oxidizing enzymes normally present in milk and the development of oxidized flavor in milk. Dorn (6) stated that a peroxide action upon the milk fat could be traced to the action of xanthine oxidase and ascorbic acid oxidation. He concluded that riboflavin was mainly responsible for the development of oxidized flavor; whereas, ascorbic acid was only secondarily so. Greenbank *et al.* (11) suggested that xanthine oxidase could be of great significance in problems of oxidative deterioration of milk and milk products. Recently, it has been reported (2) that an enzyme is involved in the development of spontaneously oxidized flavor and that the enzyme so involved may be xanthine oxidase. Studies were initiated, therefore, to determine the role of xanthine oxidase in oxidized off-flavor development.

EXPERIMENTAL PROCEDURE

Milk. The milk used during the course of the study was obtained from the college herd. Care was taken to prevent metal contamination and exposure of the milk to the direct rays of the sun. The samples were stored in glass containers at approximately 5° C. and were examined periodically for flavor development.

Enzyme preparations. Xanthine oxidase was isolated from fresh raw cream according to the method of Ball (3). Lactoperoxidase was prepared from fresh whole milk by the method of Elliott (8). A commercial beef liver catalase preparation, Paul-Lewis Laboratories, was used.

Methods. The spectrophotometric method proposed by Dunkley and Jennings (7) was used to measure the development of oxidized flavor. Essentially, it consists of measuring the intensity of color, as optical density, resulting from the reaction between the oxidized flavor components and 2-thiobarbituric acid at a

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wave length of 535 mμ. Hereafter, this method will be referred to as the T.B.A. method. The results reported are the means of duplicate determinations and are representative of several trials. As a guide for the interpretation of the data presented, the following T.B.A. scale in optical density readings was used: Slight oxidized flavor, values equal to or greater than 0.055; pronounced oxidized flavor, values greater than 0.070. Xanthine oxidase activity was determined manometrically in air at 37° C., by the method of Ball (3). Activity is expressed in terms of microliters of oxygen absorbed per hour per milliliter of milk, readings during the first 30 min. being employed. Lactoperoxidase activity was measured colorimetrically by the method proposed by Aurand *et al.* (1). Activity is expressed in terms of P.Z. units.[2] Catalase activity was measured by the titrimetric method of Maehley (18). Essentially, this method measures the disappearance of hydrogen peroxide by titration with potassium permanganate. A unit of activity is such that hydrogen peroxide is decomposed at a rate of 12 mg. per minute.

Reagents: (a) folic acid, (b) hypoxanthine, (c) *p*-chloromercuribenzoate (PCMB), (d) 2-amino-4-hydroxy-6-formyl pteridine[3] (6-PA).

RESULTS

General. The results of an earlier study (2) indicated that an enzyme was involved in the development of spontaneous oxidized flavor. It was also suggested that the enzyme involved may be xanthine oxidase. As a result, individual samples of milk were taken from cows in the college herd and were examined both for xanthine oxidase activity and for the flavor defect. Examination of the accumulated data indicated a good correlation between enzyme activity and oxidized flavor development. It was observed that when milks possessed xanthine oxidase activity ranging between 120 and 140 units, flavor development was rapid; whereas, milk possessing an activity less than 100 units failed to develop spontaneous oxidized flavor. Milks possessing an activity between 100 and 120 units were not consistent in the production of an oxidized flavor.

Relationship of xanthine oxidase activity to the development of oxidized flavor. As a result of the observations made in the preliminary study, experiments were designed to ascertain the relative significance of enzyme activity to flavor development. Herd milk was divided into three portions. One portion served as a control, one portion was heated at 63° C. for 30 min., and one portion was heated at 76° C. for 30 min. The xanthine oxidase activity of these portions, after treatment, was 75, 45, and 0 units, respectively. The milks were then subsampled. One portion served as a control, another sample was adjusted to a xanthine oxidase activity level of 138 units, and to another was added a like amount of heat-inactivated xanthine oxidase. All samples were then checked

[2] The activity of peroxidase, P.Z., is expressed in terms of the milligrams of the purpurogallin, i.e., it is the number of milligrams of purpurogallin formed per milligram of enzyme preparation, when it acts upon 5 g. of purpurogallin in 2.1 of water plus 10 ml. of 0.05% hydrogen peroxide for 5 min. at 20° C.

[3] Furnished through the courtesy of Dr. W. W. Williams, Lederle Laboratories Division, American Cyanamid Company.

at zero, one, three, and six days for flavor development by the T.B.A. method. The results of this study are presented in Table 1. It will be seen that flavor development (O.D. > 0.055) occurred only in the samples that were adjusted for enzyme activity. The results obtained for the adjusted heated milk (Sample 8)

TABLE 1

Relationship of xanthine oxidase to the development of oxidized flavor as measured by the thiobarbituric acid method

	Sample No.	Xanthine oxidase added to milk	Xanthine oxidase activity	Days			
				0	1	3	6
			($\mu l.O_2$ uptake/hr/ml)	(O.D.)			
Raw	1	−	75	.018	.020	.030	.028
	2	+	138	.029	.050	.062	.060
	3	+(Inactive)[a]	75	.020	.024	.028	.030
Pasteurized, 63° C. for 30 min.	4	−	45	.017	.020	.025	.028
	5	+	138	.025	.052	.059	.063
	6	+(Inactive)	45	.024	.025	.032	.036
Heated, 76° C. for 30 min.	7	−	0	.024	.025	.030	.026
	8	+	138	.032	.065	.070	.068
	9	+(Inactive)	0	.028	.027	.035	.034

[a] Xanthine oxidase preparation was inactivated by heating at 76° C. for 30 min., then added at the same rate as the active enzyme preparation.

were unexpected, because xanthine oxidase, an aerobic dehydrogenase, utilizes molecular oxygen as a hydrogen acceptor forming hydrogen peroxide. However, previous studies (2) indicated that hydrogen peroxide, when added to an enzyme-inactivated milk (76° C. for 30 min.), had no effect upon flavor development. Furthermore, the heated milk samples used in this study showed no measurable peroxidase activity; thus, the oxidation could not arise through the action of hydrogen peroxide (which would be expected to be formed in Sample 8) and peroxidase on an oxidizable substrate. Consequently, the effect exhibited by xanthine oxidase alone raises the question of the nature of the hydrogen acceptor involved.

Multiple enzyme systems vs. single enzyme in the development of oxidized flavor. Although there appeared to be a good correlation between xanthine oxidase activity and the development of spontaneous oxidized flavor, it was deemed necessary to consider the possibility of alternate pathways for flavor development. For example, peroxidase in the presence of hydrogen peroxide catalyzes the oxidation of many organic compounds, including ascorbic acid. Too, catalase in the presence of high substrate concentrations of low molecular weight alcohols and low peroxide concentration exhibits peroxidatic activity. Essentially, the same experimental design was used as in the foregoing experiments, except that lactoperoxidase was used as well as xanthine oxidase. The results of this study are presented in Table 2. It will be seen that the level of xanthine oxidase activity correlated well with flavor development; whereas, peroxidase activity had little or no effect on flavor development. Also, it was

wave length of 535 mμ. Hereafter, this method will be referred to as the T.B.A. method. The results reported are the means of duplicate determinations and are representative of several trials. As a guide for the interpretation of the data presented, the following T.B.A. scale in optical density readings was used: Slight oxidized flavor, values equal to or greater than 0.055; pronounced oxidized flavor, values greater than 0.070. Xanthine oxidase activity was determined manometrically in air at 37° C., by the method of Ball (3). Activity is expressed in terms of microliters of oxygen absorbed per hour per milliliter of milk, readings during the first 30 min. being employed. Lactoperoxidase activity was measured colorimetrically by the method proposed by Aurand *et al.* (1). Activity is expressed in terms of P.Z. units.[2] Catalase activity was measured by the titrimetric method of Maehley (18). Essentially, this method measures the disappearance of hydrogen peroxide by titration with potassium permanganate. A unit of activity is such that hydrogen peroxide is decomposed at a rate of 12 mg. per minute.

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			(μl.O_2 uptake/hr/ml)	(O.D.)			
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	2	+	138	.029	.050	.062	.060
	3	+(Inactive)[a]	75	.020	.024	.028	.030
Pasteurized, 63° C. for 30 min.	4	−	45	.017	.020	.025	.028
	5	+	138	.025	.052	.059	.063
	6	+(Inactive)	45	.024	.025	.032	.036
Heated, 76° C. for 30 min.	7	−	0	.024	.025	.030	.027
	8	+	138	.032	.065	.070	.068
	9	+(Inactive)	0	.028	.027	.035	.034

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were unexpected, because xanthine oxidase, an aerobic dehydrogenase, utilizes molecular oxygen as a hydrogen acceptor forming hydrogen peroxide. However, previous studies (2) indicated that hydrogen peroxide, when added to an enzyme-inactivated milk (76° C. for 30 min.), had no effect upon flavor development. Furthermore, the heated milk samples used in this study showed no measurable peroxidase activity; thus, the oxidation could not arise through the action of hydrogen peroxide (which would be expected to be formed in Sample 8) and peroxidase on an oxidizable substrate. Consequently, the effect exhibited by xanthine oxidase alone raises the question of the nature of the hydrogen acceptor involved.

Multiple enzyme systems vs. single enzyme in the development of oxidized flavor. Although there appeared to be a good correlation between xanthine oxidase activity and the development of spontaneous oxidized flavor, it was deemed necessary to consider the possibility of alternate pathways for flavor development. For example, peroxidase in the presence of hydrogen peroxide catalyzes the oxidation of many organic compounds, including ascorbic acid. Too, catalase in the presence of high substrate concentrations of low molecular weight alcohols and low peroxide concentration exhibits peroxidatic activity. Essentially, the same experimental design was used as in the foregoing experiments, except that lactoperoxidase was used as well as xanthine oxidase. The results of this study are presented in Table 2. It will be seen that the level of xanthine oxidase activity correlated well with flavor development; whereas, peroxidase activity had little or no effect on flavor development. Also, it was

TABLE 2

A study of the relationship of enzymes to oxidized flavor development as measured by the thiobarbituric acid method

Sample	Enzyme added	Xanthine oxidase activity	Peroxidase activity	Days			
				0	1	3	6
		(μl. O_2 uptake/hr/ml)	*(P.Z. units $\times 10^{-3}$)*	*(O.D.)*			
Raw	Control	51	104	.025	.025	.026	.025
	Xanthine oxidase	139	104	.035	.058	.063	.070
	Peroxidase	51	158	.033	.035	.040	.042
	Xanthine oxidase plus peroxidase	139	158	.034	.052	.060	.072
Pasteurized, 63° C. for 30 min.	Control	49	100	.026	.026	.027	.030
	Xanthine oxidase	138	100	.031	.056	.060	.065
	Peroxidase	49	160	.024	.027	.028	.031
	Xanthine oxidase plus peroxidase	138	160	.024	.056	.062	.070
Heated, 76° C. for 30 min.	Control	0	0	.025	.025	.025	.025
	Xanthine oxidase	143	0	.030	.050	.063	.072
	Peroxidase	0	154	.028	.028	.030	.032
	Xanthine oxidase plus peroxidase	143	154	.030	.052	.065	.070
	Xanthine oxidase plus peroxidase plus catalase[a]	143	154	.030	.055	.062	.068

[a] Added at the rate of 0.2 units/ml of milk.

noted that the addition of catalase to a heated milk sample which contained added xanthine oxidase and peroxidase had no effect on the flavor development. Thus, it was concluded that neither peroxidase nor catalase was involved in the direct pathway for development of spontaneous oxidized flavor.

The effect of inhibitors upon the development of spontaneous oxidized flavor. In view of the implications that only xanthine oxidase was involved in the development of spontaneously oxidized flavor, a study was made of the effect inhibitors of xanthine oxidase would have upon flavor development.

(a) Substrate inhibition. Many enzymes show inhibition by the substrate, but it appears that xanthine oxidase is exceptional, in that this inhibition occurs at unusually low substrate concentrations ($10^{-4}M$ or lower) (5). Hofstee (13), in studying the mechanism of substrate inhibition, reported that it was a non-competitive type of inhibition. In view of these facts, varying levels of hypoxanthine were added to milks that were adjusted for xanthine oxidase activity. Raw, pasteurized (63° C. for 30 min.), and enzyme-inactivated (76° C. for 30 min.) milks were used. The results of this study are presented in Table 3. It will be noted that an oxidized flavor developed in samples which were adjusted for xanthine oxidase activity and that did not contain added substrate. In contrast, the controls, unadjusted for xanthine oxidase activity, and the samples containing varying levels of hypoxanthine, failed to develop the flavor defect.

(b) Competitive inhibition. Among the effective inhibitors of xanthine oxidase are the pteridine derivatives (12, 14, 17). These inhibitors block the purine

TABLE 3
Effect of substrate upon development of oxidized flavor as measured by the thiobarbituric acid method

	Sample No.	Xanthine oxidase activity	Hypoxanthine added	O.D. at end of 6 days
		(μl. O_2 uptake/hr/ml)		
Raw	1 (Control)	103	0	.032
	2	131	0	.062
	3	131	4.4×10^{-7}	.030
	4	131	7.4×10^{-7}	.030
Pasteurized, 63° C.—30 min.	1 (Control)	94	0	.031
	2	131	0	.067
	3	131	4.4×10^{-7} *M*	.038
	4	131	7.4×10^{-7} *M*	.032
Heated, 76° C.—30 min.	1 (Control)	0	0	.032
	2	131	0	.070
	3	131	4.4×10^{-7} *M*	.040
	4	131	7.4×10^{-7} *M*	.037

and aldehyde dehydrogenating prosthetic groups of xanthine oxidase (16) and, thereby, lend themselves to a study of the role of xanthine oxidase in spontaneously oxidized flavor. Two inhibitors, 6-PA and folic acid, were used in this study. Herd milk was enzyme-inactivated by heating at 76° C. for 30 min. Then it was cooled and adjusted for xanthine oxidase activity. The milk was then subsampled; one portion served as the adjusted control and the others were treated according to the experimental design (Table 4). Flavor development was followed by use of the T.B.A. method. A summary of the data is presented in Table 4. The inhibitors were effective in preventing the development of the flavor defect and, hence, were viewed as inhibiting at the dehydrogenase site. It will be noted that 6-PA exerted a greater inhibitory effect than did folic acid. This is in agreement with an observation made by Kalckar *et al.* (14), who reported that (6-PA) on a molar basis is 200-400 times more active as an inhibitor of xanthine oxidase than is folic acid.

TABLE 4
Effect of inhibitors of xanthine oxidase upon the development of oxidized flavor as measured by the thiobarbituric acid method

Type of inhibition	Sample	Inhibitor added	Concentration	Days 0	1	3	6
				(O.D)			
	Unadjusted control[a]	0	0	0.028	0.030	0.030	0.032
	Adjusted control[b]	0	0	0.030	0.052	0.062	0.070
Competitive	1	6-PA	5×10^{-6} *M*	0.030	0.034	0.038	0.042
	2	Folic Acid	10^{-4} *M*	0.030	0.048	0.057	0.065
	3	Folic Acid	10^{-3} *M*	0.028	0.038	0.045	0.053
	4	Folic Acid	2×10^{-3} *M*	0.032	0.030	0.030	0.030
Noncompetitive	5	PCMB	10^{-4} *M*	0.027	0.045	0.058	0.065
	6	PCMB	10^{-3} *M*	0.030	0.032	0.035	0.035

[a] Xanthine oxidase activity = 75 units.
[b] Xanthine oxidase activity = 138 units.

(c) Noncompetitive inhibition. Although the above study on substrate inhibition was a study dealing essentially with a noncompetitive type of inhibition, it was deemed necessary to consider another noncompetitive inhibitor (e.g., PCMB). A summary of the data is shown in Table 4. This inhibitor, likewise, was effective in preventing flavor developmnt.

DISCUSSION

The results obtained from the above studies show: (1) The development of spontaneously oxidized flavor could be correlated with xanthine oxidase activity, (2) a single enzyme rather than a multiple enzyme system was involved in the flavor defect, and (3) known inhibitors of xanthine oxidase were effective in preventing flavor development. Thus, it was concluded that the occurrence of a spontaneously oxidized flavor in milk was dependent upon a high level of xanthine oxidase activity. Furthermore, it was concluded that the use of an enzyme inhibitor (e.g., a pteridine derivative) would enable one to control the occurrence of spontaneously oxidized flavor.

Xanthine oxidase is usually considered as an aerobic dehydrogenase, i.e., it reacts directly without the intervention of intermediary hydrogen carriers. The results obtained from the study of multiple enzymes as compared to a single enzyme indicated that only a single enzyme was involved. These observations, coupled with the observation that the flavor defect occurred in the enzyme-inactivated milk to which a crude preparation of xanthine oxidase had been added (Sample 8—Table 1), gave rise to the question of the role of hydrogen peroxide formed because of xanthine oxidase activity. In an earlier study (2), it was reported that the addition of preformed hydrogen peroxide to enzyme-inactivated milk had no effect on the development of oxidized flavor. Conversely, hydrogen peroxide when added to susceptible raw milk at low concentration (25 γ/ml) gave rise to a pronounced oxidized flavor; whereas, a higher concentration (50 γ/ml) inhibited flavor development. Chance (4), in discussing hydrogen peroxide reactions with hemoproteins, suggested that small concentrations of hydrogen peroxide may be disposed of by peroxidatic enzyme action and larger concentrations may bring into play the catalytic action. Keilin and Hartree (15) reported that peroxidatic enzyme action was dependent on a continuous supply of hydrogen peroxide, such as generated by a dehydrogenase. Therefore, since hydrogen peroxide was effective only in the presence of an enzyme, and only in a limited concentration, it would seem reasonable to conclude that xanthine oxidase exerted a peroxidatic action.

The peroxidatic action of xanthine oxidase may be explained on the basis of a free radical mechanism. One of the prevalent views regarding oxidation-reduction reactions in aqueous solutions is that the reactions involve free radicals and proceed by means of a chain mechanism. Xanthine oxidase is a metallo-flavoprotein enzyme which contains iron and molybdenum as the metal ions. These ions are essential to the action of the enzyme and are reversibly oxidized and reduced during the normal course of the enzymatic reaction. Recently, Fridovich and Handler (9) have proposed a structure for the active site of xanthine oxidase

which includes a ferric-mercaptide group as an essential part. In the normal course of reaction, electrons pass from the substrate to the first flavin and, thence, via the ferric-mercaptide groups, which are single electron acceptors; then, to the second flavin, from which they may pass to oxygen and other acceptors. Thus, the chief role of the iron ion is to make possible a very rapid electron transfer, i.e., the reversible change between the divalent and trivalent state. It is thought this explanation for the active site of xanthine oxidase can be used to explain the peroxidatic action of the enzyme by the following scheme:

$$\text{(a)}\quad 1\,e^- + H^+ \longrightarrow E\,Fe^{+++} \longrightarrow HE\,Fe^{++}$$

$$\text{(b)}\quad HE\,Fe^{++} + O_2 \longrightarrow E\,Fe^{+++} + HO_2$$

$$\text{(c)}\quad 1\,e^- + H^+ + HO_2 \longrightarrow H_2O_2$$

$$\text{(d)}\quad HO_2 + \text{oxidizable substrate} \longrightarrow \text{oxidized flavor}$$

As indicated in the hypothesis above, the uncomplicated oxidation of a substrate by xanthine oxidase results in the formation of hydrogen peroxide (a), (b), and (c); whereas, the intervention of a substance oxidizable by the transitory radical, HO_2, would result in reaction (d). Thus, according to this view, an intermediate product of hydrogen peroxide formation is the oxidant rather than hydrogen peroxide per se. Consequently, there is no need for a peroxidase in the system. The results presented herein fit this hypothesis. Furthermore, this hypothesis is substantiated by the work reported by Green and Mazur (10), in which they describe a similar reaction involving xanthine oxidase.

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WHEY PRESERVATION BY HYDROGEN PEROXIDE

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SUMMARY

Concentrations of 0.02% H_2O_2 in cheese whey exerted a preservative effect for as long as ten days. Bacterial plate counts were made of peroxide-preserved wheys of pH 4.9, 5.9, and 6.9 kept at 25, 37, and 50° C. According to these data, H_2O_2 addition shortly after separation of the whey from curd is recommended. Peroxide addition to grossly contaminated wheys of 2.8×10^7 microorganisms per milliliter resulted in a 97% bacterial kill within 1 hr. The 0.02% H_2O_2 concentration was relatively ineffective against greater numbers of bacteria. *Saccharomyces fragilis* yeast was propagated in peroxide-preserved whey from which the peroxide had been removed by the action of catalase. Results were comparable to a control fermentation on untreated fresh whey.

Enormous quantities of liquid whey from cheese are available for utilization. The United States cheese-making industry alone produces over twelve billion pounds of whey annually (8). Although it is economically impractical to consider utilization of this entire amount, increased uses for a greater proportion of the whey are possible. Suggested outlets are many (3), ranging from returning liquid whey to the farms for livestock feeding to using it as a supplemented medium in the fermentation industry.

Liquid whey remaining from the cheese-making process is composed of about 4 to 5% lactose and 0.9% protein, and constitutes a good bacterial substrate. Bacterial decomposition of this whey is a problem when storage or transportation is necessary. The use of a relatively harmless preservative may be advisable, especially if the whey is to be used for microbiological processes. In addition to being nontoxic, the preservative should be inexpensive, nonreactive with whey components, and be easily destroyed or removed when necessary.

Hydrogen peroxide has long been recommended as a dairy preservative in tropical countries. Excellent comprehensive reviews on the subject have been prepared by Rosell (10) and Lück (5). This strong and active germicide has been found acceptable in preventing souring of milk during transport and in preserving milk for cheese-making. In H_2O_2 milk-preservation methods practiced abroad, an addition of catalase is often made before the milk is ready for human consumption. This enzyme has the property of splitting H_2O_2 into its breakdown products, water and oxygen. Its use has been studied by Curran, Evans, and Leviton (2) in sporicidal action of hydrogen peroxide.

The widespread and sanctioned use of H_2O_2 as a milk preservative in some countries (9) suggested laboratory investigations into its applicability as a preservative for liquid whey. Previous studies in this area have been made by Plöttner (7), who used 0.015 to 0.03% H_2O_2 (by weight) in the preservation of

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[1] Deceased.

[2] Eastern Utilization Research and Development Division, Agricultural Research Service, USDA.

raw sweet whey and removed H_2O_2 residues by heat precipitation of the proteins or by addition of yeast. Results of our laboratory investigations on the peroxide-preservation of whey and its subsequent use for yeast growth are given in this report.

MATERIALS USED

The laboratory studies were made on liquid whey obtained from a local cottage cheese–manufacturing concern. The whey as it was received from the plant had a pH of 4.5 and temperature up to 60° C. It was transported to the laboratory in milk cans and placed in a refrigerated room at 4° C. immediately upon arrival. The insoluble proteins settled readily, and only the supernatant fluid was used for these studies.

Reagent-grade hydrogen peroxide of 28–30% purity was employed as the preservative, but data are reported on the basis of 100% purity H_2O_2. Exact composition was determined and frequently checked by Kingzett's iodine-thiosulfate titration method (11). The H_2O_2 was kept at 4° C. to prevent unnecessary deterioration due to room temperatures.

The inoculum used for testing preservation action was propagated in the laboratory at room temperature throughout this series of experiments. Sporadic feedings of both dried skimmilk and liquid whey were made to maintain its vigor. The culture consisted of an aerated sludge mixed with commercial milk that had soured. No attempt was made to identify the organisms in the mixture, except for testing their ability to attack milk and whey. An earlier investigation and isolation of the aerated sludge organisms, however, showed the predominance of *Alcaligenes, Flavobacterium, Micrococcus, Pseudomonas, Bacillus,* and *Bacterium* genera (4).

Hydrogen peroxide destruction was catalyzed by solutions of a commercial catalase preparation, Armalase A-100, a purified preparation of the enzyme manufactured by Armour Company.[2] Its potency is 100 Keil units per milliliter, one unit being the quantity needed to decompose 1 g. of 100% H_2O_2 in 10 min. at 25° C., in an inert atmosphere of CO_2 or N_2 (1).

STUDIES ON MINIMUM EFFECTIVE H_2O_2 CONCENTRATIONS

Preliminary studies were made on 50-ml. quantities of whey placed in sterile Erlenmeyer flasks having cotton plugs. Varying protective amounts of H_2O_2 were added. Half of the flasks were inoculated with the test organisms (50,000 organisms per flask); the remaining flasks were to contain only indigenous whey organisms. The flasks were held at room temperature and examined daily for physical changes in appearances. Bacterial growth was detected by transferring a loopful of the whey to sterile whey-nutrient broth solution and observing turbidity after incubation at 30° C. Results of these tests are given in Table 1 and point to 0.020% H_2O_2 as the effective level of protection. For ten days of the experiment, the broth tubes gave like turbidity readings, showing that the peroxide

[2] It is not implied that the USDA recommends the above company or its product to the possible exclusion of others in the same business.

TABLE 1

Bactericidal effect of H_2O_2 as determined by a sterility test procedure

	Age of wheys (in days)					
	1–3		4–7		8–10	
	Bacterial growth in sterile broth					
H_2O_2 in whey	I	U	I	U	I	U
(%)						
0	+	+	+	+	+	+
.010	+	−	+	−	+	−
.015	+	−	−	−	+	−
.020	+	−	−	−	−	−
.025	−	−	−	−	−	−
.030	−	−	−	−	−	−

I = Inoculated whey; U = uninoculated whey.

had checked further bacterial growth. On the 11th day, tests for residual H_2O_2 showed traces still present in all wheys. Excess quantities of sterile (Seitz-filtered) catalase were added on the 13th day to destroy the H_2O_2 and to leave the whey susceptible to possible bacterial attack. The next day, the whey solutions were reinoculated with the test organisms and reincubated at 30° C. All the flasks showed bacterial turbidity, proving that the catalase had removed all the H_2O_2.

Table 2 gives results of a test in which 1-liter amounts of whey from the cheese vat were held at room temperature (22–25° C.) in glass flasks covered with beakers. These received no supplementary organisms. Standard bacterial plate counts were made on nutrient agar incubated at 37° C. for 48 hr., as used in water analyses. Only the unprotected whey showed evidence of contamination. After 2 wk., a dense growth of a fungus, presumably *Aspergillus niger,* had appeared on the surface of all three flasks. However, for six days at room temperature, these commercial wheys had been satisfactorily protected from bacterial growth.

TEMPERATURE AND PH EFFECTS ON THE BACTERIAL ACTION OF H_2O_2

The bactericidal action of 0.02% H_2O_2 concentration at different temperatures was investigated. One hundred-milliliter quantities of refrigerated cheese whey were put into sterile bottles and equilibrated at 50, 37, or 25° C. Sufficient bacterial inoculum obtained from well-aerated sludge culture was added to give 1,000 organisms per milliliter of whey. One milliliter of 2% H_2O_2 was added, resulting in a 0.02% concentration in the final whey. At 10-min. intervals,

TABLE 2

Preservation of fresh whey by H_2O_2 as measured by bacterial plate counts[a]

	Bacterial count per milliliter whey		
H_2O_2	First day	Fourth day	Sixth day
(%)			
0	51	2,500	650,000
.020	0	0	0
.025	0	0	0

[a] 48-Hr. growth on nutrient agar at 37° C.

samples were removed for plate count determinations on nutrient agar medium. The bacterial colonies were counted after 48 hr. of incubation at 37° C.

Figure 1 shows the destruction attained at the three temperatures, as plotted on a semilog scale. Most rapid killing was observed at 50° C. Here, the high initial temperature alone had reduced the bacterial count from over 1,000 organisms per milliliter to 230. Within 5 min., 75% of the remaining organisms was destroyed by the H_2O_2. At 37 and 25° C., similar, but not such marked, effects of H_2O_2 were noted. Seventy-five per cent destruction was obtained within 13 min. at 37° C. and within 24 min. at 25° C. At none of these temperatures did the 0.02% H_2O_2 concentration achieve 100% destruction.

Table 3 summarizes the combined effects of temperature, pH, and 0.02% H_2O_2 on the viability of organisms in whey solutions. Natural whey of pH 4.9 and wheys adjusted to pH 5.9 and 6.9 with NaOH were heated to indicated temperatures and inoculated to contain 1,000 organisms per milliliter. After removing the samples for initial bacterial counts, peroxide was added. Samples were removed after 10 and 20 min. for bacterial counts. High temperatures and low pH had an immediate destructive effect, particularly at pH 4.9 and 50° C., destroying over 99% of the original organisms. The 25 remaining colonies were almost all yeasts and fungi, forms somewhat refractory to peroxide treatment as indicated by the 68% kill figure. In spite of the different beginning temperatures, pH's, and variations in viable organisms, maximum destruction occurred in all cases within 10 min. after peroxide addition. Samples taken even after an exposure of 1 hr. to peroxide showed practically no further destruction of the microorganisms.

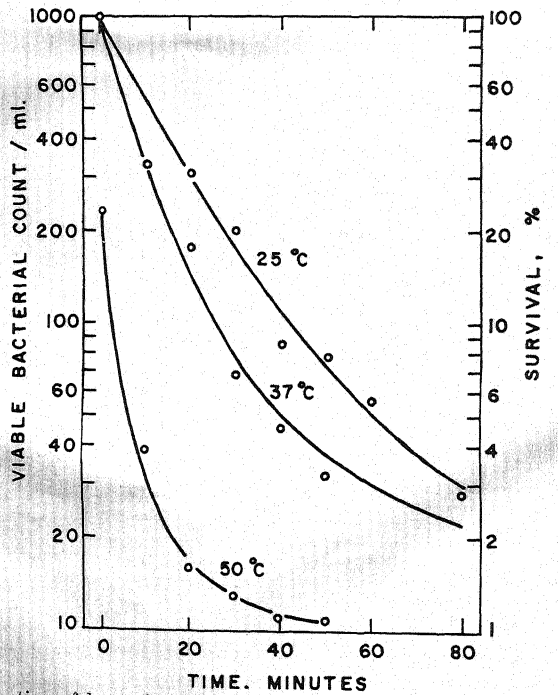


FIG. 1. Destruction of bacteria at 25, 37, and 50° C., as plotted on a semilog scale.

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TABLE 3

Bacterial destruction in inoculated whey treated with 0.02% H_2O_2 as affected by temperature and pH

Time	25° C.		37° C.		50° C.	
(min.)	*(No/ml)*[a]	*(%)*	*(No/ml)*[a]	*(%)*	*(No/ml)*[a]	*(%)*
			pH 4.9			
0	537		817		25	
10	35	(93)	24	(97)	8	(68)
20	25	(95)	18	(97)	8	(68)
			pH 5.9			
0	842		931		349	
10	56	(93)	35	(96)	9	(97)
20	34	(96)	32	(96)	6	(98)
			pH 6.9			
0	1,000		1,035		573	
10	181	(82)	61	(94)	8	(98)
20	61	(94)	64	(94)	4	(99)

Figures in parentheses represent percentage of bacteria killed, based on actual zero-hour figures. Percentage kill would be greater if based on initial inoculation of 1,000 organisms per milliliter.

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

BACTERICIDAL POTENCY OF 0.02% H_2O_2

Test organisms were used in these laboratory studies in concentrations of 1,000 organisms per milliliter of whey for these reasons: first, heated wheys received from the cheese manufacturer numbered from zero to about 400 bacteria per milliliter; secondly, 1,000 bacteria per milliliter furnished a convenient number for making bacterial plate counts without further dilution. The destructive effect of 0.02% H_2O_2 on large numbers of bacteria as may occur in contaminated whey remained to be determined. For this work, a bacterial culture was propagated from a contaminated commercial yoghurt preparation and from soured milk, and was considered representative of dairy contaminants. Bacteria numbered 2.8×10^9 in the undiluted culture. Five tenfold dilutions were made in whey. Hydrogen peroxide was added to each flask to give a 0.02% final concentration.

Table 4 presents the viable plate counts and per cent destruction achieved by 0.02% H_2O_2 acting on ranges of bacteria from 10^9 to 10^4. This concentration effected 97% destruction of 2.8×10^7 bacteria after 1 hr. of contact. After 24 hr., a 99% kill was measured. Apparently, the peroxide effect is completed within the 1st hr., as seen also with the other dilutions. The lower percentage of destruc-

TABLE 4

Destruction of bacteria in contaminated whey by 0.02% H_2O_2

Original count[a]	After 1 hr.	Kill	After 24 hr.	Kill
(per ml.)		*(%)*		*(%)*
2.8×10^9	3.6×10^9	0	2.7×10^9	Negligible
2.8×10^8	1.8×10^8	36	2.1×10^8	25
2.8×10^7	0.087×10^7	97	0.023×10^7	99
2.8×10^6	0.0085×10^6	99+	< 10	99+
2.8×10^5	0.00035×10^5	99+	< 10	99+
2.8×10^4	0	100+	< 10	99+

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

tion that occurred with the higher concentration of cells may be explained by the probable presence of catalase, elaborated by greater numbers of catalase-producing organisms. The effect of the small amount of peroxide could be quickly negated, and the bacteria would continue multiplying.

PROPAGATION OF YEAST IN PEROXIDE-PRESERVED WHEY

The effect of peroxide treatment on whey used for subsequent propagation or fermentation was investigated. One liter of liquid whey was treated with 0.02% H_2O_2 and held uncovered at room temperature (24° C.) for 24 hr. Excess catalase was added (30 units of Armalase) and allowed to stand for 30 min. Spot-testing with potassium iodide showed that all the peroxide was gone. This whey was used as part of a growth medium for the propagation of the yeast, *Saccharomyces fragilis* (12). A control fermentation with untreated whey also was run. Data compiled during these fermentations are compared in Table 5. After 3 hr., both fermentations were terminated. The harvest was about the same, and only minor differences were observed between the two fermentations.

DISCUSSION

The 0.02% H_2O_2 concentration showed both a bactericidal and bacteriostatic action against 1,000 bacteria added per milliliter of whey, and inhibited bacterial growth for as long as ten days. Traces of peroxide were easily and completely removed by the enzymatic action of subsequently added catalase, leaving the solution again susceptible to microbial attack. Satisfactory removal was demonstrated also by the successful propagation of yeast in cottage cheese whey preserved with peroxide.

Optimum conditions for peroxide treatment were suggested in the study on temperature and pH effects. Waste whey is available from cottage cheese–making operations at temperatures up to 60° C. at a pH of about 4.5 These conditions are not favorable for bacterial growth. On cooling to only 37° C., however, this whey becomes increasingly attractive to bacteria. Peroxide addition is advisable, therefore, as soon as the whey is collected.

The rate and degree of bacterial destruction by peroxide is dependent on the numbers and types of organisms and on the concentration of peroxide used. The bacterial mixture used in our experiments constituted a stringent test. This culture contained bacteria, yeast, and fungi ordinarily associated with aerobic dairy waste treatment (4). It should be noted that although 0.02% H_2O_2 accomplished up to 98% destruction of these test organisms within as little as 10 min. (at 50° C. and pH 6.7), complete destruction of all growth was

TABLE 5

Propagation of *Saccharomyces fragilis* in fresh and 0.02% H_2O_2-treated wheys

Time	Yeast count		Dry weight		Lactose		pH	
	Fresh	Treated	Fresh	Treated	Fresh	Treated	Fresh	Treated
(hr.)	*(× 10⁶/ml)*		*(mg/ml)*		*(mg/ml)*			
0	1,450	2,010	12.96	13.14	39.6	40.2	5.5	5.5
2	3,540	3,460	27.99	28.10	17.6	19.6	5.8	5.95
3	5,380	4,380	36.32	36.04	2.2	3.5	5.2	4.85

not attained. After the rapid initial killing, the peroxide effect seemed to slacken. In other tests with this same mixture of organisms, even 24 hr. of exposure to 0.02% H_2O_2 did not show 100% reduction in bacterial count. Varying degrees of resistance and indifference to the preservative are expected in such a mixture of microorganism. The susceptible species, primarily the Gram-negative forms, are annihilated; others, such as the Gram-positive rods, are unaffected and continue to proliferate. More potent concentrations of peroxide, although more destructive, were observed to precipitate and modify the whey proteins.

The bactericidal limitations of a 0.02% final concentration of H_2O_2 on milk-souring types of organisms were investigated. Within 1 hr., a 97% kill of 2.8×10^7 bacteria was effected, a remarkable achievement considering that this number of bacteria represents a grossly contaminated material. Studies showing 100% destruction of milk microorganisms have been reported by Nambudripad (6), who found 16 hr. of exposure to 0.05% H_2O_2 at 37° C. necessary for full destruction of *Bacillus megatherium* in broth solutions. This contrasted greatly to the much shorter time of 30, 40, and 45 min. required for 100% killing of *Aerobacter aerogenes*, *Alcaligenes viscosus*, and *Escherichia coli*, respectively, all Gram-negative and peroxide-sensitive forms.

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WHEY AS A SOURCE OF PLANT NUTRIENTS AND ITS EFFECT ON THE SOIL[1]

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SUMMARY

The possibility of the use of whey as a source of plant nutrients was investigated through laboratory analysis and field studies. A ton of whey was found to contain about one dollar's worth of nitrogen, phosphorus, and potassium, plus significant amounts of sodium, calcium, magnesium, and chlorides.

Whey applications do not have a detrimental effect on the pH of soils which are well limed and near neutral. However, whey added to soils with a pH of 5.0–5.5 may temporarily increase the soil acidity to a point which is injurious to plant growth.

Alfalfa will tolerate a limited amount of whey but does not benefit much from it. About one-half acre inch per week is the maximum quantity tolerable. Grasses appear more tolerant, and whey applications increased the growth of bluegrass, especially during the second growing season. This delayed benefit seems due to the slow breakdown of nitrogen compounds in the whey.

Whey benefits soil aggregation, with the amount of aggregation being dependent on the amount of whey added. However, exceedingly large applications may result in a reduction in the formation of aggregates.

Approximately 11 billion pounds of whey are produced annually in the United States. Of this total, nearly one-third occurs during the 2 or 3 mo. of the year when its production is at a maximum and its use as animal feed is at a minimum. Not only does this waste result in the loss of considerable quantities of valuable animal and plant food, but also causes a severe disposal problem.

Few research workers have studied the possibility of using whey as a soil amendment. However, in 1923, Berry (2) presented a thorough study on the utilization of whey in Scotland. In a greenhouse experiment using infertile sandy soil, he found that whey had a beneficial effect on the growth of oats. Where whey was applied on pasture at the rate of 2¼ tons per acre per day from June until September for several years, the only apparent effect was greater growth of the coarser grasses. Berry concluded that whey was beneficial to the soil but that hauling costs would probably be too high to make it profitable in most cases.

In a study of the nutritive value of whey applied to plants grown in the greenhouse on Miami silt loam, Cain (3) found that oats which received a single 100-ton-per-acre application of whey at time of planting were initially stunted, but recovered and gave a greater yield than those which did not receive whey. On a treatment that received continuous applications of whey which totaled 200 tons per acre, over a 2.5-mo. period, the oats were stunted during the early stages of growth and recovered in the later stages only when the whey applications

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were reduced. It appeared that even though whey was beneficial, in the long run very heavy and continous applications would temporarily inhibit plant growth. In a field experiment, Cain (3) applied whey on bluegrass and quackgrass sod in an effort to confirm the results he had obtained in the greenhouse. Although he concluded an increase in yield could be seen for each addition of whey, he felt that with the late fall or early spring applications, little nitrogen in the whey is released to the plants during the early part of the following growing season.

This project involved the establishment of a maximum level at which whey can be applied to field crop and the effects on crop growth and soil structure.

RESULTS AND DISCUSSION

Whey composition. Whey samples were collected at seven different times from April to November, 1957, at the Madison Milk Producers Cooperative Dairy, Madison, Wisconsin. The samples were largely a composite of whey from Swiss and American cheese, and a small amount from cottage cheese. Each sample was run in triplicate.

Method of analysis. An aliquot of 50 ml. of whey was evaporated to dryness on a 95° C. hot plate. The samples were then placed in a muffle furnace and ignited at 500° C. for 3 hr. Calcium, magnesium, potassium, and sodium were determined by extracting with .4 *N* HCl and analyzing on the Beckman Flame Photometer. Phosphorus was analyzed on the same sample, using the vanadomolybdate yellow color method (5). Nitrogen was analyzed on a 5-ml. aliquot by the Kjeldahl procedure as modified by Jackson (5). Chlorides were determined on a 10-ml. aliquot of the raw whey, which was brought to 50 ml. in volume with distilled water. About 0.5 ml. of saturated potassium chromate was used as an indicator and the sample titrated with 0.282 *N* silver nitrate (6).

The analysis given in Table 1 indicates that whey has sufficient quantities of the elements used in plant growth to be of value as a soil amendment. The nitrogen, phosphorus, and potassium in one ton of whey is worth about one dollar, based on current fertilizer prices.

TABLE 1

Analysis of separated whey from Swiss and American cheese, collected at the Madison Milk Producers Cooperative Dairy from April to November, 1957

Sample No.	Elements						
	Ca	Mg	Na	K	P	N	Cl
	(*p.p.m.*)						
1	300	50	280	1,650	400	1,400	1,000
2	350	50	650	1,750	450	1,200	1,200
3	300	60	400	1,700	450	1,200	1,150
4	300	50	480	1,800	500	1,500	1,150
5	300	50	300	1,600	500	1,350	1,000
6	350	60	800	1,600	450	1,400	1,250
7	300	50	400	1,650	400	1,500	1,200
Average	327	53	471	1,679	450	1,364	1,136

Sodium and nitrogen appear to be the two elements with the greatest amount of variation. This variation may be due to a particular type of process being used in cheese manufacture. The quantity of nitrogen may vary, depending on the amount of protein and other nitrogen-containing milk constituents left in the whey. The sodium content varies with the amount and type of salting process being used.

Effect of whey on soil pH. It has long been recognized that soil pH is one of the important factors affecting plant growth. Therefore, the effect of an acid whey solution on the soil pH was determined.

Two soil types were used, a Miami silt loam (forested soil derived from limestone glacial till), with an original pH of 6.8 and a Spencer silt loam (forested soil derived from granitic glacial till), with an original pH of 5.2.

For each type of soil, 560 g. was placed in a 600-ml. beaker and then brought to field moisture capacity by adding a combination of whey and water. The proportion of whey and water was varied to represent whey application ranging from 0 to 160 tons per acre. The pH of the whey was 4.0 at the time of application. Following the application of whey, pH readings were taken at the time intervals shown in Table 2.

TABLE 2

Soil pH resulting from various rates of application of whey on Miami and Spencer silt loam soils after various time intervals

Hours after initial application	Spencer silt loam[a]				Miami silt loam[b]			
	Whey applied in tons per acre							
	0	40	80	160	0	40	80	160
2	5.22	4.75	4.65	4.56	6.74	6.41	6.20	5.90
4	5.21	4.84	4.69	4.57	6.73	6.25	6.32	5.95
8	5.08	4.75	4.65	4.55	6.78	6.18	6.28	5.59
12	5.23	4.86	4.75	4.57	6.78	5.87	6.15	5.60
24	5.28	5.02	4.86	4.61	6.80	5.95	6.25	5.45
48	5.43	5.19	5.07	4.90	7.08	7.00	6.68	5.65
72	5.34	5.29	5.16	4.97	6.81	7.10	7.10	5.56
98	5.42	5.32	5.15	4.95	6.82	7.38	7.38	6.10
192	5.49	5.44	5.25	5.20	6.75	6.95	6.95	7.10

[a] Initial soil pH = 5.2.
[b] Initial soil pH = 6.8.
[c] pH of whey at time of application, 4.0.

As indicated in Table 2, the drop in soil pH and the length of time that the decrease persisted appeared to be dependent upon the quantity of whey added. The magnitude of the change appeared to be nearly equal in both soils. In the neutral Miami soil, the addition of whey does not cause the soil pH to drop to a point which would be injurious to plants. However, in the strongly acid Spencer soil the addition of whey may temporarily lower the pH to a point injurious to the plant metabolism.

After the whey was added to the Miami soil, there was a gradual increase in soil pH which eventually exceeded the pH of the control. This may be explained by decomposition of nitrogenous compounds releasing ammonia.

Field study on bluegrass. The first experiment was designed to indicate the residual effect and fertility value of whey on bluegrass sod.

Original whey and/or fertilizer applications to the experimental plots were begun by Cain (3) on Miami silt loam soil in November, 1955, and April, 1956. The effects of the whey and fertilizers on the growth of bluegrass during the first and second growing season following application are reported in Table 3.

There was no difference in bluegrass yields due to fall versus spring applications of whey and/or fertilizer. However, there was a significant increase in bluegrass yield for the 80 tons per acre of whey, or 40 tons of whey fortified with 240 and 1,040 lb. per acre of nitrogen supplied as ammonium hydroxide. The results also indicate that during the two growing seasons following the application of 80 tons of whey, bluegrass yields were comparable to the yields from the application of 930 lb. of 12-12-12, or about 100 lb. of nitrogen in the form of ammonium nitrate, urea, sodium nitrate, or solution nitrogen. Plots which received only nitrogen gave a good yield the first growing season, which also carried over into the second growing season. This indicates that not all the fertility value of the whey is immediately available after application, but that appreciable benefits may be derived during the second growing season, as well.

Field study on alfalfa. The boundaries of the alfalfa field plots were corrugated steel (9 in. by 12 ft.) driven 5 in. into the ground. Thrice-weekly applications, usually on alternate days, applied from 64 to 384 tons of whey or water per acre per week. The control treatment received 128 tons of water per week to approximate soil moisture conditions of the whey plots. To compare the plant nutrients in the whey with those in fertilizers, 1,000 lb. of 10-10-10 per acre was broadcast previous to any liquid applications. In one treatment, the carbon-nitrogen ratio of the whey was lowered from about 35 : 1 to less than 20 : 1 by adding ammonium nitrate. This brought the carbon-nitrogen ratio more in

TABLE 3

Residual effect of whey and/or fertilizer applications on bluegrass the first and second growing seasons after application. Bellefontaine silt loam, Gugle Farm, Madison, Wisconsin

Treatment per acre	First growing season yield[a]	Second growing season yield[b]	Average
Control	2,225[c]	2,029[c]	2,127[c]
20 Tons whey	2,295	2,459	2,377
40 Tons whey	2,830	2,861	2,846
80 Tons whey	3,310	3,821	3,566**
40 Tons whey plus 80 lb/acre of N (NH_4OH)	2,880	3,069	2,975
40 Tons whey plus 240 lb/acre of N (NH_4OH)	4,020	3,580	3,800**
40 Tons whey plus 1,040 lb/acre of N (NH_4OH)	2,380	3,794	3,087**
930 lb. 12-12-12	4,460	2,661	3,560**
112 lb. nitrogen as NH_4OH	2,800	2,075	2,438
700 lb. $NaNO_3$	4,200	2,695	3,448*
267 lb. as urea	4,030	2,398	3,214*
340 lb. as NH_4NO_3	4,530	2,673	3,602**
350 of "Uran"	4,630	2,560	3,595**

* Least significant difference at the 5% level—1,040 lb.
** Least significant difference at the 1% level—1,390 lb.
[a] Harvested in June, 1956.
[b] Harvested in June, 1957.
[c] All yields given in pounds dry matter per acre.

balance with the 10:1 soil carbon-nitrogen ratio. During the experiment, about 600 lb. of ammonium nitrate and 180 tons of whey were applied to this treatment. The results of these applications are shown in Table 4.

The soil pH was 6.7 and the alfalfa was about 6 in. high when the first applications were made on July 5, 1957. When the plots were harvested at the end of the 3rd wk., most of the plants were dead on plots receiving 128, 192, and 384 tons per acre per week. Strong odors had resulted from the 384-ton treatment, whereas only slight odors could be noticed from the rest. Only plants receiving 64 tons of whey per acre per week appeared normal, with only a slight loss and yellowing of some of the leaves near the soil surface. The pH of the plow layer was not appreciably affected by any of the treatments. The tissue samples were dried, weighed, and yields calculated on the dry weight basis as shown in Table 4.

Due to a whey shortage and an extended dry period, resumption of the treatments was impossible and the regrowth was slow except for a luxuriant growth of volunteer pigeon grass on the 128- and 192-ton treatments. No growth of any kind occurred on the 384-ton treatment.

TABLE 4

Influence of whey, water, and/or fertilizer on growth of second-crop alfalfa, and formation of soil aggregates. University Hill Farm, Madison, Wisconsin

(Whey applied July 5 through July 24, 1957)

Liquid and amount applied per wk.	Total liquid applied	Fertilizer applied	Yield-lb. dry matter per acre[a]	Aggregates in each size fraction[c]			
				2.0 mm.	1.0 mm.	0.5 mm.	0.25 mm.
(tons/acre)	*(tons/acre)*			*(%)*			
128 Water	294	None	3,052	3.89	6.60	14.80	34.72
64 Water	181	1,000 lb. 10-10-10	3,624	4.81	7.49	15.36	34.18
64 Whey	181	None	2,622	9.36	14.0	23.82	42.90
64 Whey	181	72 lb. NH_4NO_3 weekly	2,857	10.08	15.27	25.83	44.58
128 Whey	378	None	[b]	12.30	19.81	33.00	51.81
192 Whey	512	None	[b]	10.00	16.11	28.24	46.30
384 Whey	768	None	[b]	5.99	10.59	22.55	40.97

[a] Average of four replicates.
[b] Most plants dead at end of treatment.
[c] Sampled May 7, 1958.

The yield of alfalfa was reduced with the whey treatment, in spite of the addition of the ammonium nitrate. Therefore, it appears that alfalfa can tolerate only about 0.5 acre inch per week without harm, and that quantities in excess of this will result in killing and stunting of many of the alfalfa plants. This may be the result of the extreme demands put on the oxygen supply of the soil when large quantities of easily decomposable organic matter are added. However, the heavy growth of the pigeon grass on the 128- and 192-ton treatments indicates that a shallow root system would be more satisfactory. It appears that grasses are more tolerant to large whey applications, and that whey will have residual benefits on bluegrass even during the second growing season.

While conducting a greenhouse study on soil aggregation, Chesters (4) used various materials such as whey, sucrose, corn stover, cow manure, sawdust, and

various peats. He found that a single application of 150 tons per acre of whey gave the maximum per cent of soil aggregates over 0.25 mm. in diameter. Soil aggregates may be defined as naturally occurring clusters of soil particles which promote soil tilth, drainage, and aeration, and increase water-holding capacity and reduce erosion.

To obtain information under field conditions, soil aggregation analyses were made on samples taken about 10 mo. after whey applications were made. The analysis was made by using the wet sieving method devised by Yoder (7) and modified by van Bavel (1), to determine the percentage of water stable aggregates which is indicative of desirable soil structure. The results are given in Table 4 and indicate that all levels of whey application helped to form water-stable aggregates. The number and size of aggregates increased with each increment of whey to the 192-ton treatment, after which there was a decrease approximately to the level of the 64-ton treatment. At the time of sampling for aggregate analysis (May, 1958), no regrowth had occurred on the 384-ton treatment; whereas, the remainder had a mixture of alfalfa and grass. The lack of cover on the 384-ton plots may well have caused a breakdown of the aggregates during the winter freezing and thawing and the rainfall impact during the spring rains.

CONCLUSION

The present study indicates that whey can improve soil structure and provide plant nutrients, but it appears that feeding to animals would accomplish more complete utilization of whey solids. However, at periods of maximum production, when adequate disposal means are unavailable, the application of whey to soil can benefit the crops and soil, where care is exercised in its application.

ACKNOWLEDGMENT

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GENETIC AND ENVIRONMENTAL INFLUENCES UPON SOLIDS-NOT-FAT CONTENT OF COW'S MILK [1]

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SUMMARY

Monthly composite milk samples from the Holstein and Guernsey herds of the New Jersey Agricultural Experiment Station were taken from July, 1953, to February, 1958. Solids-not-fat (SNF) content was determined by formulas based on specific gravity (Watson Pattern Lactometer) and fat percentage (Babcock fat test). A study of 4,772 samples from 222 cows revealed no specific yearly trends in SNF content, although 1954 was significantly lower with Holsteins, and 1956 significantly higher with Guernseys, than the averages of the remaining years. Month effects were significant and similar for both breeds. SNF content was high in December and January and low in June and July. Although stage-of-lactation effects were more extreme on Holsteins, SNF content of both breeds was high shortly after parturition, dropped to a lactation low at 40 to 60 days, increased very slowly to 6 mo., and then increased rapidly to 10 mo. Pregnancy effects were noticeable at the 4th or 5th mo. of gestation, and accounted for most of the SNF increase in the latter stages of lactation. Two types of lactation averages were calculated: (1) based on all tests from parturition to 305 days, and (2) based on the second through sixth tests. Records from 182 cows by 50 sires were studied. SNF content of 310 Holstein records averaged (1) 8.48 and (2) 8.42%; 110 Guernsey records averaged (1) 8.95 and (2) 8.93%, respectively. SNF content decreased with age in a curvilinear manner. Intra-cow repeatabilities were (1) 0.61 and (2) 0.56, showing the 305-day record (1) to be slightly more desirable. Heritability estimates calculated from paternal half-sib correlations were 0.93 and 0.97. Doubling the intra-breed dam-daughter regression coefficient obtained from 87 dam-daughter pairs resulted in a heritability estimate of 0.57.

Owing to the decrease in market value of butterfat in recent years, greater emphasis has been placed on the solids-not-fat (SNF) content of milk in the United States and many other countries. This gradual change has focused more attention on the factors which affect SNF content. Although there have been numerous studies on this subject, the majority was based on laboratory procedures which by nature did not lend themselves to the assay of mass data by field techniques. This investigation was undertaken to measure and evaluate the influence of several environmental and genetic factors upon SNF content of milk as determined by procedures applicable to field use.

Over a period of several years, downward trends in SNF content in England were noted by Bailey (7) and by Provan and Jenkins (48); conversely, an increase was indicated in the Netherlands by Janse (30). Little systematic change in either direction was found in England by Baker and Cranfield (8) or in Scotland by Waite *et al.* (60). Long-time trends were often difficult to evaluate because of changes in assay procedures. In nine English herds studied by the

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Milk Marketing Board (38) over a 4- or 5-yr. period, the smallest range in yearly averages was 0.08%, the largest was 0.34%. Both were Holstein herds.

A review of 14 investigations by Herrmann (26) showed changes in average monthly SNF percentages of varying degree. There, and in other studies throughout the world, SNF content was shown to be lowered by high temperatures (14, 28), low nutritive plane (6, 39, 53, 60), and drought (22, 33, 49), or combinations of these factors (11, 12, 18, 23, 25, 29, 32, 35, 37, 38, 48, 57, 58). With optimum conditions, it appeared that SNF content increased. Results of the individual studies varied according to local conditions.

Changes in SNF content during the lactation also were described by Herrmann (26) in a review of nine investigations. In general, percentage dropped from an initial high level shortly after parturition to a lactation low between 1 and 2 mo., remained generally constant for 5 to 6 mo., and gradually increased during the remainder of the lactation. Similar trends have been described by others (5, 15, 18, 25, 38, 46, 55, 57, 60). The effect of month of lactation was found to be statistically significant by Nicholson *et al.* (40) with 18 Holsteins over a 3-yr. period. On more limited data, dissimilar lactation trends were observed (35, 51). To remove the confounding effects of month of year on stage of lactation, Waite *et al.* (60) obtained estimates of each effect independent of the other. The common stage-of-lactation curve still remained.

Investigating the effect of pregnancy, Bartlett (9) and Rowland (54) noted increases between the 4th and 5th mo. of gestation which accompanied the characteristic decline in milk yield. In farrow cows, SNF percentage seemed to decrease slightly as the lactation progressed. Bailey (5) noticed pregnancy effects after the 4th mo. for mature cows and slightly later for first- and second-calf heifers. Tests from 6 wk. to 6 mo. were recommended as being the most indicative of SNF percentage for investigational work. Gorrie and Harvey (23) found stage of pregnancy to be the largest of several factors measured. Effects were apparent after 100 days.

In seven investigations reviewed by Herrmann (26), SNF percentage was shown to decline with age at a rate varying between 0.03 and 0.18% per lactation. Further confirmation was available from Bailey (4), Edwards (18), Flux *et al.* (19), and White and Drakely (64). Gowen (24) noted that decreases due to age were linear, and amounted to 0.024% per year. A decrease of about 0.05% per lactation was noted by Waite *et al.* (60), and 0.10% by Griffiths and Featherstone (25) and the Milk Marketing Board (38).

Relatively few heritability estimates have been reported. Several investigations reviewed by Tyler (59) indicated a positive genetic influence. Most comprehensive of those reviewed were the investigations of Robertson *et al.* (50), who estimated the heritability of SNF content to be 0.53, and Politiek (47), who estimated it to be between 0.5 and 0.7. Shaw and Fourt (55) and the Milk Marketing Board (38) also presented evidence of genetic influence. By doubling the intra-sire daughter-dam regression from 146 pairs of two breeds, Johnson (31) calculated heritability estimates of 0.35 and 0.34. The repeatability of SNF content of consecutive lactation records was reported by Wilcox *et al.* (65)

to be 0.80 when data from 70 cows of two breeds were appropriately combined. Flux *et al.* (19) estimated repeatability of 495 records of 161 cows to be 0.75.

INVESTIGATIONAL PROCEDURE

Determinations on a few selected cows were made as a preliminary step from July through September, 1953. Routine samples were subsequently taken on every cow of the Holstein and Guernsey herds of the New Jersey Agricultural Experiment Station on or about the 15th of each month. By February, 1958, SNF content had been determined in 4,772 samples from 222 cows. Unweighted night and morning samples were combined. Although slight differences in SNF content between night and morning samples have been suggested by several (3, 9, 15, 17, 21, 22, 23), these differences were not always considered important and were not always in the same direction. No significant differences were found by several others (20, 24, 32, 37, 41, 45, 57, 58).

Until June, 1955, SNF percentage was measured by subtracting fat percentage, as determined by the Babcock fat test, from total solids percentage, as determined from A.O.A.C. tables for Quevenne Lactometer readings (2). Subsequently, a Watson Pattern Lactometer (62) was used and SNF percentage calculated by subtracting fat percentage from total solids percentage as determined from USDA tables (61). Comparison of estimates of SNF percentage by the two methods on milk of the New Jersey Agricultural Experiment Station resulted in a correlation of +0.92 (65), with no difference in the mean values. The superiority of the Watson Pattern Lactometer was demonstrated by Robinson *et al.* (52), but not by Cosgrove and Cobble (10) or Madden *et al.* (36).

To avoid possible confounding of month and stage of lactation due to disproportionate subclass numbers, these effects were measured independently of each other by a method shown by Patterson (43) to be comparable to the method of fitting constants on data provided by Snedecor (56).

Milk samples were taken and tested by assistants in conjunction with the regular DHIA supervisor. No samples were taken before the seventh day after freshening. Records were kept in the DHIA book such as could be a standard practice for commercial dairy farms.

Lactation averages were calculated by dividing the total pounds of SNF by the lactation milk yield. Records ranged from 260 to 305 days in length, the minimum being well within the range recommended by Politiek (47). Since tests taken from the 2nd to 6th mo., inclusive, have been shown to be quite consistent (5), because this portion of the lactation curve approximated a horizontal line, an additional average was calculated using the tests in this interval.

To measure the systematic effects of age, and to obtain estimates of repeatability and heritability, analytical procedures described by Snedecor (56) and Anderson and Bancroft (1) were utilized.

RESULTS AND DISCUSSION

Effect of year. Annual means for SNF percentage from samples taken in the years 1954-57 are presented (Table 1). Samples taken in late 1953 and early

TABLE 1
Mean SNF content of samples from four calendar years

Year	Breed					
	Holsteins			Guernseys		
	Cows	Samples	Mean	Cows	Samples	Mean
	(No.)	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*
1954	96	751	8.38*	35	289	8.90
1955	98	792	8.53	34	294	8.90
1956	104	786	8.56	28	290	9.09*
1957	96	787	8.54	37	342	8.91
Over-all	156[a]	3,116	8.50	62[a]	1,215	8.93

* Differs significantly ($P < 0.05$) from other 3 yr.
[a] Not a sum; most cows represented in several years.

1958 have been omitted, since they represented only a portion of these years. The mean values represented averages of sample percentages only. Limiting this phase of the investigation to four calendar years caused the exclusion of all data from three Holsteins and one Guernsey, as well as a number of samples from other cows; thus, 4,331 samples from 218 cows were included.

An analysis of variance of the unweighted monthly means showed significant differences among years. A major portion of this variation was due to the high year for Guernseys (1956) and to the low year for Holsteins (1954). Applied on a within-breed basis, the Duncan Multiple Range Test (16) indicated that only these years differed from the remaining three. A factor in the high 1956 Guernsey average was an exceptionally high January test (9.64%). On this test day fat percentage (5.2%) for Guernseys was also the highest for the 4-yr. period; at the same time, a 4-yr. high (8.81%) was established for Holstein SNF percentage. The low year for Holsteins (1954) seemed due to low tests in June and July. A portion of the variation could have been attributable to changes in the population over the 4 yr. However, since no attempt to select for SNF content was made, since only about 20 to 25% of the cows were replaced each year, and since the replacement daughters could be expected to have about the same inherent level as the existing population of dams, the change in SNF content caused by a change in the make-up of the herd would be expected to be very small. Therefore, the variation among years was considered to be due largely to effects other than animal differences.

No general trends upward or downward were apparent over the short period covered; the odd year was different for each breed and the shift was in an opposite direction. No attempt was made to adjust the data for year effects. It was interesting to note that even though the station Holstein herd tested higher (4.0%) for butterfat than the breed average, there was no accompanying superiority for SNF percentage.

Effect of month and stage of lactation. Analyses of month-of-year and stage-of-lactation effects were carried out with data limited to those samples collected on the first ten monthly sampling days following parturition. The first sample from each cow was taken between seven days post-partum and seven days plus 1 mo. These procedures were purposely followed to make them comparable to

DHIA practices. Of course, similar distributions of the different times at which cows were first sampled would be expected for each month of the year. Furthermore, if changes are similar to those in fat percentage, the major differences associated with this variation would be in the first month, and with a uniform distribution throughout the month, the average can be considered as representative for comparison with later samples. After excluding samples taken after the tenth sampling period, 3,139 Holstein and 1,223 Guernsey samples remained.

Several analyses of variance were carried out on each breed. The basic data were analyzed to point out the major effects, and to establish the within-subclass (error) variance estimate. As a comparative method, an analysis of variance of the unweighted means of the month by stage-of-lactation subclasses was completed. Finally, the data were analyzed after adjustment of each main effect for the other. The sources of variation, significance of mean squares, and estimates of variance and variance percentage are presented (Table 2) for the latter two analyses.

Owing to the removal of the confounding of month and stage of lactation and the nature of the analyses, the analyses of the adjusted data presented the best estimates of the factors measured. Similar significance and estimates of variance and variance percentages resulted from all three analyses. The method of unweighted means has been considered appropriate for preliminary analysis and usually adequate where disproportion of subclass numbers was not great. Because of simplicity, the method has much to recommend it. Although the subclass values ranged in number from 1 to 28 with Guernseys and from 3 to 54 with Holsteins, the added accuracy of the exact method did not seem to justify the extra effort required.

It was obvious that both month and stage of lactation independent of each other were real effects of appreciable size. The variance estimate for months was greater in the Holstein data than in the Guernsey data, but not significantly so. The variance of stage of lactation was significantly greater for Holsteins than for Guernseys. Thus, percentage-wise, the portion of the total variance attributable to months was smaller with Holsteins than Guernseys. Breed differences were noted by Davis *et al.* (13), who found that the range of serum solids percentage during the lactation was largest with Guernseys (1.08%), less with Holsteins (0.65%), and least with Jerseys (0.35%). Stage of lactation seemed to have more effect on Holsteins than Shorthorns in a study of several herds of each breed by the Milk Marketing Board (38).

The actual adjustment factors used to remove the confounding of month and stage-of-lactation effects were small. The largest factor amounted to only 0.08%; the majority were 0.02% or less. In light of the degree of accuracy of the SNF determinations, the adjustment process may not be necessary, or even justified.

The interaction between month and stage, though highly significant, accounted for a very small portion of the variance. The data were graphed, but yielded little further information of any systematic trends, such as a greater month-of year effect in early lactation than in later lactation.

TABLE 2

Variance and variance percentages for month and stage-of-lactation effects as determined by two methods of analysis

Source of variation	Holsteins					Guernseys				
	d.f.	Unweighted means		Adjusted data		d.f.	Unweighted means		Adjusted data	
		(V.)	*(%)*	*(V.)*	*(%)*		*(V.)*	*(%)*	*(V.)*	*(%)*
Month	11	0.244**	30	0.203**	27	11	0.126**	41	0.114**	41
Stage of lactation	9	0.367**	46	0.353**	48	9	0.044**	14	0.035**	13
Interaction	99	0.016**	2	0.011**	1	99	0.020**	7	0.009**	3
Residual	3,019	0.174	22	0.174	23	1,103	0.118	38	0.118	43

** Significant at the 1% level.

Error variation seemed greater among Holstein SNF estimates; comparison of the residual terms showed the difference to be highly significant. It must be pointed out, however, that with 1,103 and 3,019 degrees of freedom, even very small differences can be highly significant. The residual was composed of various random effects of which the variation among animals would seem to be the largest (65). It was assumed in this investigation, as in each of the investigations previously reviewed, that differences among animals would not be confounded with stage-of-lactation and month-of-year effects in such a way as to introduce any important bias. Such a bias would have been possible had cows with unusually high or low SNF percentages happened to fall in subclasses with very small numbers, but subclasses with small numbers did not seem to be out of line with subclasses with larger numbers. Furthermore, since the average calving interval in this herd was about 13 mo., many cows in a given stage of lactation would not be represented in the same month of the year in successive lactations.

Graphic portrayals of month (Figure 1) and stage-of-lactation (Figure 2) effects are presented. Month effects were generally similar for both breeds, with SNF content moving in the same direction at the same time. An exception was the change from August to September, which was downward for Guernseys and slightly upward for Holsteins. Depending on local environmental conditions, various investigators have reported the most important causes of month effects to be temperature, feed, or drought. With this population, rainfall was adequate during the period investigated; a combination of changes in temperature and feed was probably responsible for the month effects.

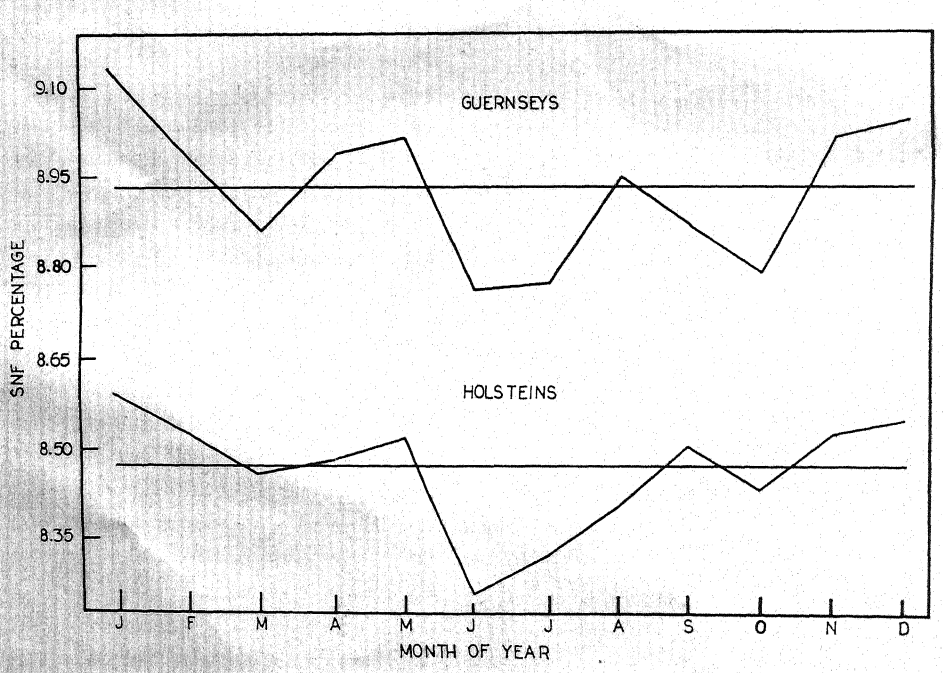


FIG. 1. Effect of month on SNF content.

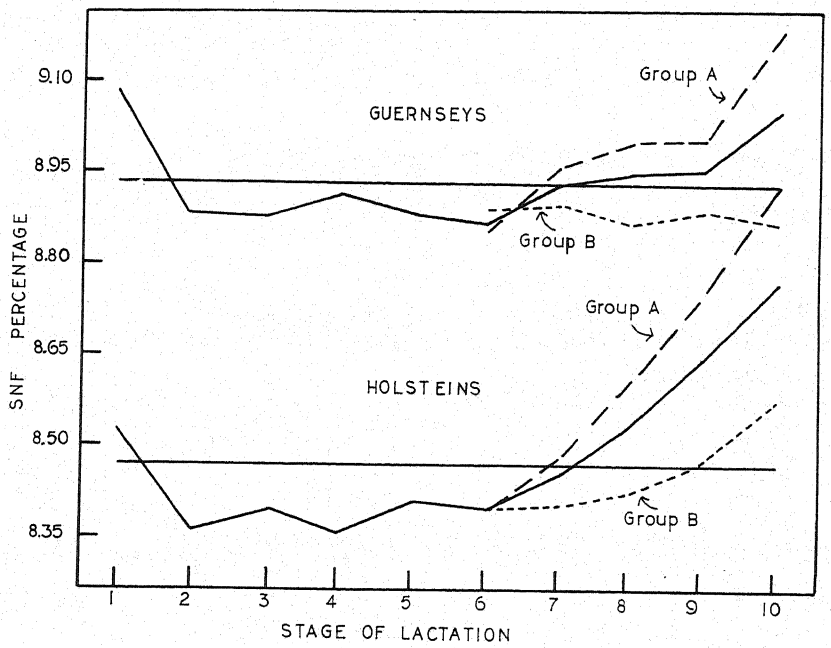


FIG. 2. Effect of stage of lactation on SNF content.

The stage-of-lactation curve was similar to those presented in the literature. The initial high value, the steep decline, leveling off, and gradual climb seemed to be independent of breed, location, and investigator.

Effect of gestation. Samples taken from the sixth through tenth test period of each lactation were divided into two groups for each breed. Group A consisted of samples from those animals successfully bred between 50 and 118 days postpartum, thus freshening in less than a 400-day calving interval. Cows conceiving later, or not at all, were placed in Group B. The groups were kept intact throughout, and the mean SNF content for the successive tests presented (Figure 2). The original stage-of-lactation lines were thus divided into two components according to the stage of gestation. Assuming that month effects were the same for both groups, and since the estimates for month effects had been determined from the combined data, percentages in both groups were adjusted by use of the same factors.

SNF levels for Group A and Group B animals were not appreciably different at the sixth test period for either breed. Since the average date of conception of Group A animals was probably near the midpoint between 50 and 117 days, the seventh test period corresponded to the 4th or 5th mo. of pregnancy and demonstrated a pregnancy effect similar to that found by Bartlett (9), and if all age groups were combined, by Bailey (5).

With the exception of the first milk test, where the effects of parturition contributed to high SNF content, much of the so-called stage-of-lactation effect was due to pregnancy with the Holsteins, and practically all could be so ex-

plained with the Guernseys. Further, since the make-up of Group A of each breed was similar in regard to stage of gestation, the greater stage-of-lactation effect found in the Holstein data could not be so explained. Reproductive efficiency was roughly similar for each breed, since 63% of all Holstein determinations, and 60% of all Guernsey determinations, were placed in Group A.

The Group B samples were further divided according to stage of pregnancy. This additional breakdown has not been shown because the loss of numbers made the lines more erratic and specific animal effects became apparent. Although stage of pregnancy was similar in the additional groups, it was found again that its effect was greater with Holsteins than with Guernseys. Although the differences between breeds could be suspected to be due to different management practices, this was unlikely, since the two breeds were kept at the same location with common housing, the same labor, and under a single herdsman.

Effect of age. The mean SNF content of 310 completed Holstein records was 8.48 and 110 Guernsey records 8.95%. These averages were based on all milk samples taken in the first 305 days of each lactation. Averages based on the second through the sixth milk test (five-test average) were 8.42 and 8.93%, respectively, for the two breeds. Represented in the study were 182 cows by 50 different sires.

Records were not adjusted for environmental effects. Stage-of-lactation and pregnancy effects should have been similar in consecutive lactations; if not, the second measure of average SNF content (based on the second through sixth milk test) should have eliminated their effects. Although month-of-year has been shown to account for 27 to 41% of the variation among individual samples, a lactation record based on a series of tests over an extended period of time need not necessarily be biased by month effects. Such a biasing effect could be described as a month-of-freshening effect. White and Judkins (63) believed earlier that month-of-freshening did affect the lactation average, but recent studies by Bailey (4) and the Milk Marketing Board (38), incorporating considerably more data and more sensitive statistical analyses, have indicated otherwise. Adjustments for month-of-freshening accounted for only 1% of the total variation in a study by Robertson *et al.* (50), who indicated that the mentioned adjustment was definitely not justified. On the basis of these previous reports, and the erratic nature of the month-of-year effects, records have not been adjusted for month-of-freshening for this investigation.

Records were arranged according to lactation number for both breeds and for both methods of calculation (Table 3). A gradual, though irregular, decrease of SNF content with age was noted. Preliminary study of the averages showed a departure from linearity, the cubic term being significant. Therefore, the cubic curve was calculated on an among-record-within-cow basis for each breed and for the 305-day and five-test records. The genetic differences among animals were, thus, not involved in the calculations. The constants were all significant or highly significant (Table 4), supporting the preliminary calculations. In the order listed in the table, the combined effects of the regressions represented 13, 12, 25, and 24% of the within-cow variation among SNF records.

TABLE 3

Effect of age on SNF lactation average

Lactation number	Holsteins			Guernseys		
	Records	305-day	5-test	Records	305-day	5-test
	(No.)	*(%)*	*(%)*	*(No.)*	*(%)*	*(%)*
1	80	8.68	8.63	28	9.18	9.16
2	67	8.55	8.49	18	8.96	8.92
3	51	8.43	8.35	16	8.83	8.80
4	40	8.35	8.28	16	8.89	8.84
5	28	8.38	8.31	14	8.86	8.84
6	17	8.25	8.17	9	8.85	8.86
7-10	27	8.23	8.18	9	8.85	8.80
Total or av.	310	8.48	8.42	110	8.95	8.93

TABLE 4

Constants representing the effects of age on SNF content

Type of record	Intercept	Partial regression		
		Linear	Quadratic	Cubic
Holstein				
305-day	8.72	−0.1661**	+0.0287**	−0.0015*
5-test	8.68	−0.1853**	+0.0327**	−0.0018**
Guernsey				
305-day	9.43	−0.3714**	+0.0768**	−0.0049**
5-test	9.47	−0.4237**	+0.0883**	−0.0057**

** Significant at the 1% level.
* Significant at the 5% level.

The gradual lowering of SNF content with age was in agreement with other reported investigations. None of those reviewed, however, presented conclusions of curvilinear effects. Whether the decrease was a feature of age per se, or the result of gradual deterioration of udder health or function, has not been studied. Mastitis has been shown to lower SNF content. As cows age, it may be assumed that mastitis attacks occur more frequently, each time causing some permanent damage. Some, if not all, of the so-called age effect could have been caused by progressive udder deterioration.

Repeatability and heritability. Expected SNF values for each lactation number, according to the calculated equations, were determined, and deviations from the Lactation 3 value used for adjusting all individual records. Lactation 3 was used as the adjustment base, since the mean of the lactation numbers was 3.20 and a whole number was considered desirable. Adjusted data were then analyzed separately by breed, and then combined, to give repeatability and heritability estimates. These estimates were obtained through analyses of variance, and portions of the analyses are presented (Table 5).

Repeatability, $C/(C+R)$, amounted to 0.61 and 0.56 for the 305-day and five-test records, respectively. Utilizing z transformation and correction for bias, the 95% confidence limits of these intraclass correlation coefficients were 0.52 and 0.69, and 0.46 and 0.65, respectively. Slightly more within-cow variability existed among the five-test records, but this difference was not significant.

TABLE 5

Analysis of variance of SNF records adjusted for lactation number

Source of variation	Degrees of freedom	Mean squares	Expectation of mean squares	Components of variance
		(305-day av.)		
Sires in breed (S)	48	0.266**	R + 2.389 C + 8.678 S	S = 0.018
Cows in sire (C)	134	0.105**	R + 2.257 C	C = 0.036
Records in cow (R)	236	0.023	R	R = 0.023
Total	418			
		(5-test av.)		
Sires in breed (S)	48	0.296**	R + 2.389 C + 8.678 S	S = 0.021
Cows in sire (C)	134	0.111**	R + 2.257 C	C = 0.036
Records in cow (R)	236	0.028	R	R = 0.028
Total	418			

** Significant at the 1% level.

It had been suggested (5) that the five-test records might be superior. The demonstration of parturition and pregnancy effects in this study indicated that the methods warranted comparison. If repeatability and within-cow variability were to be used as criteria, however, the 305-day record appeared to be the most desirable.

The rather high repeatability indicated that the characteristic was fixed to a considerable degree in an individual. As expected, the repeatability estimate obtained in this study, representing the intra-cow correlation of any record with any other record, was somewhat lower than an earlier reported estimate (65) of 0.80, where the intra-cow correlation of consecutive records was measured.

There seemed to be no advantage in using records based on the second through sixth milk test. Two factors appeared to be the most important causes of the lowered repeatability. First, with the nine or ten samples taken for the 305-day record, more of the random errors due to sampling and testing, etc. would balance than with five samples. Secondly, the effects of season, shown to be large and irregular through the year, would have more opportunity to equalize in the longer records. Both of these factors are important, and both might have acted to the detriment of the accuracy of the five-test average. Since the season effects have not been consistent among the various studies, it is of questionable value to attempt to adjust SNF records specifically for season.

Heritability estimates, $4S/(S + C + R)$, were high (0.93 and 0.97, respectively). The 95% confidence limits of the two correlations obtained (0.23** and 0.24**) were estimated to be 0.03 and 0.43, and 0.04 and 0.44. The problems involved in estimating the heritability of lactation SNF content were not of a simple nature. The limitations in numbers prevented more sensitive analyses. This problem was met with some success in studies of beef cattle by Patterson *et al.* (44), who felt that an illogically high heritability estimate from the intra-class correlation of paternal half-sibs could be attributed to nonisolated interactions. These investigators obtained estimates of several interactions from portions of the data which lent themselves to analysis; on this basis, the effects of major interactions were removed. Further, it was shown that the average relationship of half-sibs was closer to 0.33 than to 0.25, which led to a factor of

3, rather than 4, in the formula for estimating heritability from intra-sire half-sib correlations. Thus, it may be concluded that determinations of heritability are not too satisfactory by the half-sib method when the sample size is small.

From 87 dam-daughter pairs, an intra-breed regression coefficient of 0.28**, with 95% confidence limits of 0.04 and 0.53, was determined. Heritability measured on this basis amounted to 0.57. A number of sires were represented by only one daughter-dam pair, preventing the analysis on a within-sire basis because of the drastic reduction in degrees of freedom. Since all records were more or less contemporary, however, bias due to environmental correlation which would have been removed by an intra-sire scheme of analysis was undoubtedly small.

Lush (34) has indicated that the regression of daughters on dams is the appropriate basis for calculating heritabilities. The regression is used in preference to the correlation coefficient, primarily because of the undesirable effect of selection of the independent variable on the correlation coefficient, in this case the selection of dams. However, the correlation between daughter and dam may sometimes be justified if there has been no selection of dams for the measure being studied, as in this case. The interclass dam-daughter correlation was 0.34**, with confidence limits of 0.13 and 0.52. Doubling these values gave a heritability estimate of 0.68, with a possible range of 0.26 to 1.00.

Assuming, as stated by Lush (34), that repeatability can be used as an estimate of the upper limit of heritability, then some credence could be placed upon the heritability estimate (0.57) obtained from the dam-daughter regression. The estimates of 0.53 found by Robertson *et al.* (50) and of 0.5 to 0.7 found by Politiek (47) would then be substantiated.

CONCLUSIONS

Use of field techniques and indirect SNF determination procedures was adequate to measure the environmental factors included in this investigation. Month, stage of lactation, and pregnancy were shown to influence SNF content of individual samples significantly. Lactation SNF content decreased systematically with age in a curvilinear manner. Averages based on all tests during the first 305 days of lactation were only slightly more reliable than averages based on the 2nd through 6th mo. of lactation. Repeatability and heritability estimates for SNF lactation average were found to be higher than most commonly accepted values for lactation milk and fat yield.

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DISTRIBUTION AND INHERITANCE OF HEMOGLOBIN VARIANTS IN AMERICAN CATTLE [1]

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SUMMARY

The relative frequencies and genetic determination of the two electrophoretically distinguishable adult bovine hemoglobin types were investigated in a large sample drawn from a number of different breeds and sources. Electrophoretic studies of hemoglobins from 121 males and 646 females, representing nine cattle breeds, indicate that the Ayrshire, Holstein, Angus, Hereford, and Milking Shorthorn breeds probably have only Type A hemoglobin; whereas, the Brown Swiss, Guernsey, Jersey, and Sindhi breeds have both Type A and Type B. An analysis of hemoglobin types among progeny from parents of known types substantiates the theory that the occurrence of these two types of hemoglobin is genetically determined by a pair of codominant alleles—HbA, determining Type A, and HbB, determining Type B. Estimates of the frequency of HbB among the major dairy breeds are: Ayrshire—0.000, Holstein—0.000, Guernsey—0.116, Brown Swiss—0.117, Jersey—0.333. No gross physiological differences have been observed to be correlated with differences in hemoglobin type. The trait may be of some practical value in parentage determination and in the tracing of breed origins. The most significant feature of these results is that they represent a further demonstration of single-gene determination of a protein specificity.

Electrophoretically distinguishable hemoglobins have been observed in the blood of cattle of North Africa by Cabannes and Serain (3), of Great Britain by Bangham (1), and of the United States by Grimes, Duncan, and Lassiter (6) and by Salisbury and Shreffler (12). In each instance, two types of hemoglobin have been observed which migrate at different rates in an electrical field. Three studies (1, 6, 12) indicated probable breed differences in the relative frequencies of the two hemoglobin variants, suggesting genetic determination of the trait. In the most extensive study reported thus far, Bangham (1) presented data indicating that these two types of hemoglobin are genetically controlled by a pair of alleles at a single locus, with neither allele exhibiting any apparent degree of dominance, so that in a heterozygous individual the blood contains a mixture of the two types of hemoglobin. Salisbury and Shreffler (12) reported preliminary observations which seemed to support this theory. The present paper reports the results of a more extensive survey of cattle of a number of breeds and from a number of sources. The objective of this survey was to obtain further evidence establishing the mode of inheritance of these hemoglobin types and to obtain estimates of the gene frequencies and genotypic distributions within breeds.

EXPERIMENTAL PROCEDURE

Animals. Included in this study were 121 males and 646 females, representing nine breeds and drawn from four experiment station herds and the two

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Illinois artificial breeding centers. The breeds represented were the five major American dairy breeds plus the Sindhi, Milking Shorthorn, Angus, and Hereford breeds. The latter three breeds were represented only by bulls from the artificial breeding centers. The Holsteins, Guernseys, and Ayrshires studied were all in the University of Illinois herd, except 42 Holstein bulls and 21 Guernsey bulls, which were sampled at the two artificial breeding centers. Brown Swiss were sampled from the University of Illinois herd, the artificial breeding centers, the North Louisiana Hill Farm Experiment Station at Homer, Louisiana, and the USDA herd at Beltsville, Maryland. Jersey bloods were obtained from the same sources as the Brown Swiss, plus the Iberia Livestock Dairy Station at Jeanerette, Louisiana. The Sindhis and Sindhi crosses were obtained from the Beltsville, Homer, and Jeanerette stations.

Because previous observations (1, 6, 12) have indicated that the occurrence of fetal hemoglobin, Type F, in very young individuals may be confused with the adult Type B hemoglobin, no animals were included in this survey which were not at least 3 mo. of age. The nature of the similarities and differences between bovine hemoglobin Types B and F has been studied in detail and reported by Grimes, Duncan, and Lassiter (7).

Hemoglobin analysis. A heparinized sample of venous blood was drawn from each animal to be tested. Seven milliliters of 0.9% NaCl solution were added to a 1-ml. sample of the blood, the sample was centrifuged to sediment the erythrocytes, and the supernatant was removed. The erythrocytes were washed twice more by addition of 0.9% NaCl solution, centrifugation, and removal of the supernatant. The washed cells were then hemolyzed with a quantity of distilled water sufficient to give a hemoglobin solution of approximately 10% concentration. The prepared solutions were used either fresh or after storage at 0° F.

For analysis by electrophoresis, 0.01 ml. of the prepared hemoglobin solution was banded upon a strip of Whatman 3 MM filter paper on a Durrum hanging strip electrophoresis apparatus (2) (manufactured by Spinco). Separations were carried out at room temperature for 3 hr. in veronal buffer, pH 8.6, ionic strength 0.05, at 8 v. per cm. In the initial phases of the survey, the strips were dyed in bromphenol blue solution for 6 hr., washed in 5% acetic acid, and fixed in an acetate buffer. However, in the course of the survey, it was established that with the concentrated hemoglobin solution described, the rate of migration could be accurately determined without dyeing, by examination of the visibly colored bands of hemoglobin. Therefore, for the qualitative purposes of this survey, the dyeing was discontinued.

Recent observations indicate also that, for survey work of this type, separation of the hemoglobins from the plasma proteins is unnecessary. Consistently accurate results were obtained by diluting 1 vol. of whole blood with 3 vol. of distilled water, to hemolyze the erythrocytes, and analyzing the resulting solution by electrophoresis. The strips can not be dyed, but the position of the hemoglobin can be determined by its characteristic color, as previously discussed. No results were obtained which indicated interference in normal migration of the hemoglobins due to the presence of the plasma proteins.

RESULTS

Three specific types of results were obtained in the analysis by paper electrophoresis. One type of hemoglobin, designated Type A, migrated a mean distance of 1.5 cm. under the experimental conditions described. Another type, designated Type B, migrated a mean distance of 2.5 cm. under these conditions. A third type of result apparently arose from the presence of both Type A and Type B hemoglobins in the blood of some individuals. This result, called Type AB, was characterized by two bands of hemoglobin, one migrating 1.5 cm., the other migrating 2.5 cm. A mixture of equal quantities of blood containing only Type A hemoglobin and blood containing only Type B hemoglobin was found to give a similar result. The specific hemoglobin type of any individual is entirely repeatable, both for duplicate analyses of the same sample of blood and for analyses of samples taken at different times. Re-analysis of 35 adult individuals after intervals of a year or more revealed no instance of change in hemoglobin type.

Table 1 shows the distributions within breeds of hemoglobin types among the unselected animals analyzed. Those individuals tested as a part of the genetic study because of their presumptive or predicted hemoglobin type are excluded from these data, and only those individuals sampled at random are included. Type A hemoglobin was found among all of the breeds studied, and was found to be the sole type among the Holstein, Ayrshire, Milking Shorthorn, Angus, and Hereford individuals tested. Types B and AB were observed only in the Brown Swiss, Guernsey, Jersey, and Sindhi breeds. The 42 Ayrshires tested provide rather sound evidence that if Type B hemoglobin occurs in this breed, its frequency must be quite low. This is supported by the observations of Bangham (1), who found only Type A among 38 Ayrshire bulls, and of Grimes, Duncan, and Lassiter (6), who found only Type A among eight individuals. The absence of Type B among the large number of Holsteins tested suggests that this trait occurs very rarely, if at all, within this breed. This is also in agreement with the results of Bangham (1) and Grimes, Duncan, and Lassiter (6) from smaller numbers of animals. The numbers of Shorthorn, Angus, and Hereford bulls are much too small to suggest any hemoglobin characterization of these breeds, but the results, which show the presence of Type A only, are consistent with those of Bangham (1), which included larger numbers of individuals.

The size and the variety of sources of the sample of Brown Swiss should make it possible to characterize this breed reasonably well with respect to this trait. Approximately one-fourth of the Brown Swiss have Type AB hemoglobin, while about 1% are of Type B. The remainder are Type A. Although the sample is smaller, and probably less representative, the results for the Guernseys sampled indicate a frequency of hemoglobin types similar to that found for the Brown Swiss. The sample for the Jersey breed, drawn from five different sources, should also be reasonably representative of the breed. The analysis of this sample indicates a higher frequency of Types B and AB than found for the Guernsey

TABLE 1

Distribution of hemoglobin types within breeds

Breed	Sex	Total	Hemoglobin type		
			A	AB	B
Ayrshire	F	42	42	0	0
Brown Swiss	F	120	91	28	1
	M	17	15	2	0
		137	106	30	1
Guernsey	F	51	42	9	0
	M	22	15	6	1
		73	57	15	1
Holstein	F	112	112	0	0
	M	43	43	0	0
		155	155	0	0
Jersey	F	77	41	25	11
	M	13	3	7	3
		90	44	32	14
Sindhi	F	1	0	0	1
	M	1	0	0	1
		2	0	0	2
Milking Shorthorn	M	12	12	0	0
Aberdeen-Angus	M	9	9	0	0
Hereford	M	5	5	0	0
Crossbred					
Holstein-Guernsey, F_1	F	45	40	5	0
Holstein-Guernsey, other	F	46	46	0	0
Brown Swiss-Sindhi, F_1	F	14	0	14	0
Brown Swiss-Sindhi, other	F	46	23	21	2
Jersey-Sindhi, F_1	F	9	2	5	2
Jersey-Sindhi, other	F	37	14	17	6
Holstein-Sindhi, F_1	F	2	0	2	0
Holstein-Sindhi, other	F	9	4	5	0

and Brown Swiss breeds. Over one-half of the individuals tested were either of Type B or Type AB.

Although only two pure Sindhis were tested, both of which were Type B, some further indication of frequency of Type B hemoglobin in this breed may be obtained from the data on crosses of the Sindhi with the Brown Swiss, Jersey, and Holstein breeds. These results suggest that Type B hemoglobin is the predominant type in the Sindhi breed, and perhaps the only type, since the frequency of Type B among the F_1 crosses of Sindhi with European breeds is higher than that found in any of the European breeds involved in the crosses. Examination of data on individual crosses in which the hemoglobin type of the European dams is known reveals that in all but one instance the Sindhi sires used in the crosses were Type B, assuming that the proposed theory of inheritance of the characteristic is correct. It must be pointed out, however, that

most of the Sindhi individuals tested, or involved in the crosses the progeny of which were tested, were rather closely related. Thus, these observations may reflect the hemoglobin distribution in only a particular family or line of the breed, rather than the breed in general.

In connection with the two-allele theory of inheritance of the trait suggested by Bangham (1), the authors have proposed (12) that the genes controlling these hemoglobin types be designated as HbA, controlling the less rapidly migrating Type A, and HbB, controlling the more rapidly migrating Type B. Thus, according to this nomenclature and the suggested mode of inheritance, an individual HbA/HbA would have only Type A hemoglobin, an individual HbB/HbB would have only Type B, and a heterozygous individual, HbA/HbB, would have both Type A and Type B, the condition which has been designated as Type AB.

Table 2 contains the distributions of hemoglobin types among the offspring resulting from matings of sires and dams of known types. Included are the actual distributions observed among the offspring in each class of matings, and the distributions expected with genetic control by a pair of codominant alleles. Since the presumptive heterozygous condition occurs both in males and in females, and since the ratios among offspring of reciprocal crosses show no significant differences, the possibility of sex linkage is effectively eliminated. Therefore, in the data presented in Table 2, reciprocal crosses have been pooled. Chi-square and probability values for the A × AB crosses indicate how well the observed distribution fits the expected. The numbers of animals in the AB × AB and B × AB classes are too small for any meaningful test of fit, but it will be noted that the observed and expected distributions are quite comparable in these classes. It should also be observed that no offspring was produced in any of these crosses which was not possible under the two-allele theory of inheritance. This is of particular importance in the critical tests, A × A, B × B, and A × B, in which all of the progeny of a given mating class must have identical genotypes. Larger numbers among the crosses involving Types B and AB would, of course, be desirable. The numbers are limited because the low frequency of individuals with these types among the breeds from which most of the data were drawn makes such matings infrequent.

In Table 3 may be found similar data for a number of instances in which the hemoglobin type of only one parent was known. While the small number of animals in the AB and B groups, and the division of these animals among several breeds, makes a test for the fit of the ratios of questionable reliability, values are included for a Chi-square test. The distribution of genotypes among the unknown parents was estimated from the breed gene frequencies in Table 4. Expected distributions of progeny were calculated on this basis. The Chi-square and probability values for these groups indicate that the observed distributions do not show deviations from the expected distributions which can not be attributed to chance. Also, it should again be observed that no offspring occurred among these groups which could not possibly occur under the proposed mode of inheritance.

TABLE 2

Distribution of hemoglobin types among offspring of parents of known type

Parental types	Value	Total	Hemoglobin type A	AB	B	χ^2	Probability
A × A	Obs.	78	78	0	0		
(HbA/HbA × HbA/HbA)[a]	Exp.	78	78	0	0	.....	.....
A × AB	Obs.	45	27	18	0		
(HbA/HbA × HbA/HbB)	Exp.	46	23	23	0	1.778	0.20>P>0.10
AB × AB	Obs.	3	1	2	0		
(HbA/HbB × HbA/HbB)	Exp.	4	1	2	1	.....	.....
A × B	Obs.	4	0	4	0		
(HbA/HbA × HbB/HbB)	Exp.	4	0	4	0	.....	.....
B × AB	Obs.	5	0	3	2		
(HbB/HbB × HbA/HbB)	Exp.	6	0	3	3	.....	.....
B × B	Obs.	5	0	0	5		
(HbB/HbB × HbB/HbB)	Exp.	5	0	0	5	.....	.....

[a] Presumptive genotype.

If the theory of inheritance is correct, the phenotypic distributions of hemoglobin types shown in Table 1 are also genotypic distributions, and the frequencies of the genes controlling these types may be calculated from these data. Because there were apparent differences in the frequencies and distributions between males and females within breeds having both hemoglobin types, the distributions of males and females within these breeds were first tested separately by the Chi-square test for homogeneity of a binomial distribution (13). This test showed the separate distributions of Brown Swiss, Guernsey, and Jersey males and females to be homogeneous with respect to their own frequencies, although the probability that the deviations in Jersey females were due to chance was very close to the 0.05 level. The differences in frequencies between males and females within breeds were then tested (13). It was found that none of these differences was significant. Therefore, gene frequencies and the test for equilibrium of the breed populations were calculated for the pooled samples of males and females within breeds. The gene frequencies were calculated in the usual manner for a two-allele trait without dominance (10), and are presented in Table 4. If random mating is assumed, the expected genotypic array may be calculated from these frequencies and compared with the observed genotypic distribution in the usual test for equilibrium of a population (10). The Chi-square and probability values given in Table 4 for the three breeds in which both hemoglobin types occur indicate no significant departure in the observed frequencies from those expected with random mating in a two-allele system.

DISCUSSION

The evidence in Tables 2 and 3 is consistent with that obtained by Bangham (1), upon which the theory of genetic control was originally based, and serves to support and strengthen that hypothesis. This mode of inheritance is also consistent with findings with respect to hemoglobin variants in humans (9), sheep (4), and mice (5), although codominance in the genetic determination of mouse hemoglobins has not yet been demonstrated.

TABLE 3

Distribution of hemoglobin types among offspring of one parent of known type

Parental type	Value	Total	Hemoglobin type			χ^2	Probability
			A	AB	B		
A × ?	Obs.	116	97	19	0		
(HbA/HbA)[a]	Exp.	116	101	15	0	1.225	0.30>P>0.20
AB × ?	Obs.	25	6	13	6		
(HbA/HbB)	Exp.	26	9	13	4	2.000	0.50>P>0.30
B × ?	Obs.	9	0	5	4		
(HbB/HbB)	Exp.	9	0	5	4	0.000	P=1.00

[a] Presumptive genotype.

While it can not be considered as direct or definite evidence, the hypothesis is also supported by the fact that the tests of breed populations imply equilibrium conditions under this type of genetic control. It must, of course, be recognized that certain factors, such as nonrandom mating, selection, and lethal effects, might influence this test for equilibrium. Three explanations may be suggested for the positive test for equilibrium: (1) The theory of inheritance is correct and, with respect to this trait, mating is relatively random with no important lethality or selection. (2) The theory of inheritance is correct, but the effects of lethality, selection, and nonrandom mating act in different directions, in such a way that by chance the test for equilibrium is positive. (3) The theory of inheritance is not correct, but chance combinations of lethal effects, selection, and nonrandom mating have resulted in a phenotypic array which fits the two-gene hypothesis.

Certainly, selection and nonrandom mating are more the rule than the exception in dairy cattle breeding. However, since the trait can be recognized only by the methods previously described, no direct selection based upon hemoglobin type can have been employed in the animals sampled, nor can any departure from random mating have been based upon this trait. Observations in the Illinois station herd have revealed no gross physiological differences within breeds between animals having different types of hemoglobin. Therefore, it does not seem likely that any extensive selection for hemoglobin type due to genetic correlations with other traits has taken place. Also, if either type of hemoglobin caused lethal effects, this would be expected to result in deviations among the ratios of genotypes in the data in Tables 2 and 3. On the basis of these considerations, the first explanation seems the most probable, although the others can not be excluded. It would appear, however, that the evidence presently available dictates the acceptance of the hypothesis of genetic determination by a pair of codominant alleles, until such time as new evidence might disprove it.

It is not presently known whether these observations are of practical significance. These hemoglobin variants could be of some value in parentage determination in certain breeds. Their occurrence and frequency might be useful in tracing breed origins and relationships among certain breeds. Present evidence indicates some common ancestry for the Brown Swiss, Guernsey, Jersey, and Sindhi breeds. Information on the hemoglobin characterization of other Euro-

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TABLE 4

Gene frequencies and genetic equilibria within breeds

Breed	q_{HbB}[a]	Value	HbA/HbA	HbA/HbB	HbB/HbB	χ^2	Pobability
Ayrshire	0.000	Obs.	42	0	0		
		Exp.	42	0	0	.....	.....
Brown Swiss	0.117 ± 0.031[b]	Obs.	106	30	1		
		Exp.	107	28	2	0.652	0.50>P>0.30
Guernsey	0.116 ± 0.053[b]	Obs.	57	15	1		
		Exp.	57	15	1	0.000	P=1.00
Holstein	0.000	Obs.	155	0	0		
		Exp.	155	0	0	.....	.....
Jersey	0.333 ± 0.069[b]	Obs.	44	32	14		
		Exp.	40	40	10	3.600	0.10>P>0.05

[a] q_{HbB} = gene frequency of HbB.
[b] ± limits of 95% confidence interval.

pean, African, and Asian breeds could be useful in this respect. As previously stated, no gross within-breed differences in health, vigor, livability, conformation, or production, which could be related to differences in hemoglobin type, have been observed in those animals tested in the University of Illinois herd. It is possible, however, that differences could occur which would be observed only under specific physiological or environmental stresses which "trigger" some physiological failure, as in the case of sickle-cell and other anemias arising from hemoglobin variants in humans (14).

The quantitative investigations of Grimes, Duncan, and Lassiter (6), showing equal concentrations of the two hemoglobin types in what has been shown here to be the heterozygous condition, provide an interesting example of genetic dosage effects. Each allele apparently has equal efficiency with regard to the quantity of hemoglobin produced, but the electrophoretic differences indicate that the molecular structures of the two hemoglobins are slightly different. Thus, these two alleles have quantitatively equal, but qualitatively different, effects. That electrophoretic differences reflect structural differences in the hemoglobin molecule has been conclusively demonstrated by the work of Pauling *et al.* (11), and the later work of Ingram (8). This difference and the simple genetic determination of it are the most significant features of these results. They constitute further evidence supporting the role of genes as determinants of protein specificities.

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ASSOCIATIONS BETWEEN SERVICE INTERVAL, INTERVAL FROM FIRST SERVICE TO CONCEPTION, NUMBER OF SERVICES PER CONCEPTION, AND LEVEL OF BUTTERFAT PRODUCTION

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SUMMARY

The interval from first service to conception decreases as the service interval increases from 0 to 127 days, at which it reaches a minimum. After a service interval of 127 days, the interval from first service to conception increases slightly as the service interval increases. The length of the service interval accounts for only 1.4% of the variance of the interval from first service to conception; thus, even though its effect on the interval from first service to conception is significant, its effect is small and of little importance.

Service interval does not significantly affect the number of services per conception and accounts for only 0.3% of the variance of services per conception. In general, the number of services per conception *(Y)* decreases linearly as the service interval *(X)* increases, the partial regression of Y on X for a constant butterfat production being -0.0034 ± 0.0023.

The partial regressions of the number of services per conception and the interval from first service to conception on butterfat production for a constant service interval are $+0.003 \pm 0.001$ and $+0.091 \pm 0.046$, respectively, and are significant at the 0.05 level of probability. The partial regression of the number of services per conception on butterfat production for a constant service interval and constant interval from first service to conception is -0.00004 ± 0.0007 and is not significant. It is concluded that there is no real biological relationship between services per conception and level of butterfat production.

The service interval alone accounts for 16.8% of the variance of the interval from calving to conception. The interval from calving to conception increases at an increasing rate as service interval increases to approximately 50 days. After a service interval of 50 days, the interval from calving to conception increases almost linearly and in an approximate 1 to 1 ratio as the service interval increases.

Calving interval has approximately the same relationship to service interval as has the interval from first service to conception. With the present data, the average service interval must be from 36 to 49 days to maintain an average calving interval of approximately 365 days. If the conception rate was increased to 60%, the average service interval allowed could be increased approximately 11.5 days, and a calving interval of approximately 365 days could still be maintained.

The association between the interval from calving to the first service (service interval) and the number of services per conception has been investigated by a number of workers (6, 11, 12, 19, 21, 23, 24, 25). In general, these workers reported that the services required per conception decreased as the service interval increased to 100–120 days, when the number of services per conception reached a minimum. VanDemark and Salisbury (25), recognizing the importance of the length of the calving interval, suggested that cows should first be serviced 60 to 80 days after parturition, so as to require a relatively small number of services per conception and still maintain a calving interval of approximately 12 mo.

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Trimberger (24) recommended that cows should not be serviced until 50 days or more after parturition.

Gaines (13) found a correlation of 0.039 ± .010 between the service period (the interval from calving to conception) and the first month's milk production; thus, it is evident that the first month's milk yield and service period are practically independent. In another study, Gaines and Palfrey (14) found a correlation of 0.053 ± 0.049 between the average length of the first nine calving intervals and the average yield per day over the nine calving intervals of 186 cows. On the basis of this correlation, the authors concluded that calving interval has no effect on yield and that high-yielding cows are not bred so as to freshen less frequently than low-yielding cows. Several workers (1, 3, 18, 20, 22) have reported that higher-yielding cows require more services per conception, whereas others (2, 4, 10, 13) have reported that there is no relationship between level of production and services per conception.

The associations between the service interval and the interval from first service to conception, and between the number of services required per conception and the interval from first service to conception, have not been well established.

The objectives of the present study are to determine the associations between the service interval, the interval from first service to conception, the number of services per conception, and the level of butterfat production; and to demonstrate some of the consequences of these associations.

SOURCE AND DESCRIPTION OF DATA

The data for this study were taken from the Red Danish Milkrace progeny groups at the Danish progeny testing stations during the testing year 1955–56. All heifers were in their first lactations when the data involved in this study were recorded. The observations consist of 2,056 artificial services on 902 first-calf heifers by 51 Red Danish Milkrace sires. The number of daughters per sire ranged from 14 to 20, with a mean of 17.7. Eight hundred fifty-three of the 902 heifers conceived and these heifers received a total of 1,782 services. Some of the 49 heifers returned to the farmers as nonpregnant may have been pregnant as a result of their last services, but no information on pregnancy examinations was available after the heifers left the testing stations.

The variables involved in this study are days from first service to conception *(W)*, number of services per conception *(Y)*, service interval (days from calving to first service) *(X)*, services per cow *(Y')*, and first lactation fat production in kilograms *(Z)*. The herdsmen at the various testing stations recorded the calving dates and service dates on barn sheets, and the heifers were diagnosed for pregnancy by veterinarians or technicians, who indicated the service at which conception most likely occurred. The herdsmen then recorded the date of pregnancy examination and the service at which pregnancy most likely occurred. The data in this study on days from calving to first service *(X)*, days from first service to conception *(W)*, services per conception *(Y)*, and all services *(Y')* were taken from the barn sheets. The first lactation butterfat records are those given in (15) for the Red Danish Milkrace sires.

All cows included in this study were first-calf heifers, all were given ample opportunity to conceive, there was no selection for production among the daughters of a sire, and there was no tendency to give high producers more of a chance to conceive than low producers; therefore, the data should provide unbiased estimates of the relationships between days from first service to conception, services per conception, total number of services, service interval, and butterfat production.

ANALYSIS OF DATA AND RESULTS

The service intervals ranged from 44 to 202 days; there were only 11 below 50 days and only 13 above 150 days, of which four were above 160 days. In Table 1, the mean days from first service to conception, the services per conception, and the total number of services are listed according to the length of the service interval.

The means listed under the service intervals from 44–49 days appear to be slightly larger than those listed under the longer intervals, but other than this there seems to be no consistent trend of the means with respect to the length of service interval.

In Table 2, the analyses of variance for days from first service to conception, for services per conception, and for all services are shown. In no case is the variation between ten-day service interval groups significant at as low a probability as 0.05.

In spite of the general lack of significance of the between-group mean squares, one should not conclude that the length of the service interval is not associated with the length of the interval from first service to conception, with services per conception, or with total number of services per cow. The differences associated with the different lengths of service interval could be confounded with test station differences in management and with service sire differences in fertility. Such differences are likely to be small in the data, because a concerted effort is exerted to standardize management practices at the various stations and the field nonreturn rates for the various service sires used were approximately equal. In addition to the possibility of confounded effects, it is possible that the linear or a curvilinear component or both components of the length of service interval could be significant, as was shown by VanDemark and Salisbury (25).

To avoid the possibility of confounded variables and to more precisely determine the associations of the length of service interval (X) with the interval from first service to conception (W), services per conception (Y), total services (Y'), and butterfat production (Z), the within-sire variations were studied. In addition, the square root of the service interval $\sqrt{(X)}$ was included, to help account for a curvilinear effect.

On observing the butterfat records published in (15), it was obvious that there were variations in days in production and age at calving. The age at calving ranged from 658 to 1,341 days, with a mean of 899 days, and the days in milk ranged from 232 to 370 days, with a mean of 304 days. To adjust the individual records for these variations in age at calving and days in milk, the within-sire

TABLE 1

Mean number of days from first service to conception, number of services per conception, and number of services as distributed by ten-day periods of the service interval

		Length of service interval												
		44-49	50-59	60-69	70-79	80-89	90-99	100-109	110-119	120-129	130-139	140-149	150 and up	Totals
Heifers that conceived														
Days from first service to conception	$\overline{W}$	72.1	43.29	41.14	35.77	36.35	30.04	31.07	38.96	37.00	41.54	40.00	12.42	36.08
	N	10	39	127	177	178	134	86	45	23	13	9	12	853
	σ_w	56.09	39.85	48.79	48.89	47.33	42.31	39.59	46.08	41.14	44.71	42.00	24.52	45.78
Services per conception	$\overline{Y}$	2.50	2.15	2.16	2.10	2.13	2.04	1.95	2.20	2.17	1.92	2.00	1.50	2.09
	N	10	39	127	177	178	134	86	45	23	13	9	12	853
	σ_y	1.43	1.22	1.37	1.45	1.47	1.39	1.19	1.39	1.11	0.95	1.12	0.80	1.37
Per cent conceived on first service		30.0	38.5	40.2	49.7	46.1	44.8	47.7	40.0	30.4	46.2	44.4	66.7	44.9
Heifers failing to conceive														
No. services	$\overline{Y''}$	7.00	6.60	5.78	5.75	5.80	5.50	4.33	4.00	5.50	4.67	3.00	4.00	5.53
	N	1	5	9	8	10	4	3	2	2	3	1	1	49
All heifers														
All services	$\overline{Y'}$	2.91	2.66	2.40	2.26	2.32	2.14	2.03	2.28	2.44	2.44	2.10	1.69	2.28
	N	11	44	136	185	188	138	89	47	25	16	10	13	902
	$\sigma_{y'}$	1.92	1.16	1.68	1.61	1.68	1.50	1.26	1.41	1.42	1.46	1.10	1.03	1.58
Per cent conceived on first service		27.2	34.1	37.5	47.6	43.6	43.5	46.1	38.3	28.0	37.5	40.0	61.5	42.5

TABLE 2
Analyses of variance for days from first service to conception, services per conception, and all services

Source	Heifers that conceived				All heifers	
	Days from first service to conception *(W)*		Services per conception *(Y)*		All services *(Y')* per cow	
	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Between ten-day service intervals	11	2,996	11	0.91	11	2.46
Within ten-day service intervals	841	2,084	841	1.88	890	2.50

multiple regression of butterfat production (in kilograms) on age at calving *(A)* and days in milk *(D)* was derived and is Equation (1).

$$\hat{Z} = -18.59 + 0.075\,A + 0.510\,D \qquad (1)$$

By using Equation (1), the butterfat records were adjusted to an average length of lactation of 304 days and an average age of calving of 899 days. Since the ranges in age at calving and days in milk were relatively narrow, it was assumed that a linear equation would be sufficient to adjust the butterfat records.

The within-sire correlations involving service interval *(X)*, the square root of service interval $\sqrt{(X)}$, days from first service to conception *(W)*, services per conception *(Y)*, and adjusted butterfat production *(Z)* are listed in Table 3. The correlation between services per conception and days from first service to conception is large (0.859), as would be expected, but it indicates that the two variables are not identical. By holding one of the two constant, the other would retain approximately 26% of its original variance. It is recognized that services per conception *(Y)* and days from first service to conception *(W)* are not normally distributed for a given service interval. However, judging from Table 1 it is apparent that the variances of *(W)* and *(Y)* are approximately equal for the various intervals of *(X)*. Thus, for estimating the regression coefficients the requirements that the errors be noncorrelated and have the same variance are satisfied, but for making the usual tests of significance the requirements that the errors be normally and independently distributed are not satisfied. Consequently, one should consider the standard errors of regression coefficients involving *(Y)* and *(W)* as rough approximations.

TABLE 3
Within-sire correlations between service interval *(X)*, square root of service interval ($\sqrt{X}$), days from first service to conception *(W)*, services per conception *(Y)*, and adjusted butterfat production *(Z)* for those heifers that conceived

	$\sqrt{X}$	W	Y	Z
X	0.952	−0.042	−0.047	0.082
$\sqrt{X}$		−0.073	−0.045	0.045
W			0.859	0.078
Y				0.065

The above correlations are based on 801 degrees of freedom and the 0.05 and 0.01 critical values are 0.069 and 0.091, respectively.

The interval from first service to conception. The multiple regression of the interval from first service to conception (W) on the service interval (X) and $\sqrt{(X)}$ was derived on a within-sire basis from the correlations, standard deviations, and means given in Tables 3 and 4. The resulting regression equation is Equation (2).

$$\hat{W} = 110.99 + 0.62\ (\pm\ 0.24)\ X - 13.88\ (\pm\ 4.49)\ \sqrt{X} \qquad (2)$$

$$R_{W \cdot X\sqrt{X}} = 0.116 \qquad \text{D.F.} = 800$$

Equation (2) is plotted in Figure 1. In Equation (2), the partial regression coeefficints are significant at the 0.01 level of probability. Although the above multiple correlation coefficient ($R_{W \cdot X\sqrt{X}} = 0.116$) is significant at the 0.01 level of probability, X and $\sqrt{X}$ have accounted for only 1.4% of the variance of the interval from first service to conception (W). From Equation (2), it is apparent that only a minor part of the variance of the interval from first service to conception is associated with variation in the service interval. VanDemark and Salisbury (25) found that only 1.0% of the variance of services per conception was associated with variation in the service interval. One would expect this rather close agreement, since the correlation between services per conception and the days from first service to conception as given in Table 3 is 0.859.

TABLE 4

Means and within-sire standard deviations for (X), $(\sqrt{X})$, (W), (Y), and (Z)

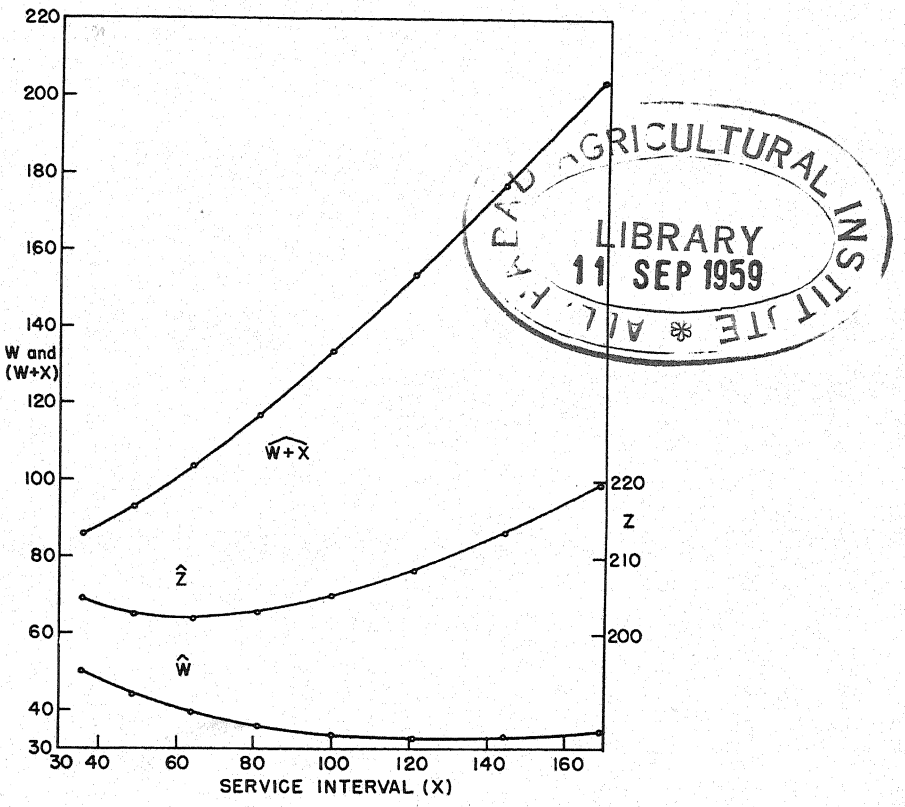
	X	$\sqrt{X}$	W	Y	Z
Mean	86.7	9.24	36.08	2.09	204.0
σ	21.4	1.14	44.84	1.39	34.7

On observing Figure 1, it is apparent that the average interval from first service to conception decreases at a decreasing rate as the service interval increases from 36 to 127 days. As the service interval increases from 127 to 169 days there is practically no change in the average interval from first service to conception. If the derivative of $\hat{W}$ with respect to X for Equation 2 is set equal to zero, the minimum average interval from first service to conception is found to occur at a service interval of 127 days. As the service interval increases from 36 to 127, 49 to 127, and 64 to 127 days, the average interval from first service to conception decreases 17.1, 11.2, and 6.6 days, respectively. Thus, it is apparent that relatively long average intervals from first service to conception are associated with service intervals below 60 days.

Since the interval from first service to conception and the service interval are both significantly correlated with butterfat production, as shown in Table 3, one should, perhaps, include Z as an independent variable along with X and $\sqrt{X}$ when predicting W. This was done, and the resulting equation is Equation 3.

$$\hat{W} = 88.51 + 0.55(\pm\ 0.24)\ X - 12.88\ (\pm\ 4.50)\ \sqrt{X} + 0.09\ (\pm\ 0.05)\ Z.$$

$$R_{W \cdot zx\sqrt{x}} = 0.136 \qquad \text{D.F.} = 799 \qquad (3)$$



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FIG. 1. The regressions of the interval from first service to conception *(W)*, the interval from calving to conception *(W + X)*, and butterfat production *(Z)* on the service interval *(X)*. *Z* is expressed in kilograms and *W*, *X*, and *W + X* are expressed in days.

$$\hat{W} = 110.99 + 0.62\,X - 13.88\,\sqrt{X}.$$

$$\widehat{W+X} = 110.99 + 1.62\,X - 13.88\,\sqrt{X}.$$

$$\hat{Z} = 245.26 + 0.69\,X - 10.90\,\sqrt{X}.$$

If Equation (3) is plotted for a constant Z of 204 kg., the resulting graph coincides almost exactly with the graph of Equation (2) in Figure 1. For corresponding regression values to differ more than a day, the service interval must exceed 160 days. Shown in Table 5 are the mean squares of the interval from first service to conception, accounted for independently by the service interval and the level of butterfat production. In general, only a small part of the variance of the interval from first service to conception is associated with variation in the level of butterfat production.

The interval from calving to conception. From the correlations, means, and standard deviations in Tables 3 and 4, the regression of the interval from calving to conception *(W + X)* on the service interval *(X)* and $\sqrt{X}$ can be derived. This regression was derived and is Equation 4.

$$\widehat{W+X} = \hat{W} + X = 110.99 + 1.62\ (\pm 0.24)\ X - 13.88\ (\pm 4.49)\sqrt{X}$$
$$R_{(W+X)\cdot X\sqrt{X}} = 0.41 \qquad \text{D.F.} = 800 \qquad (4)$$

Since service interval is a part of the interval from calving to conception, the regression of $(W + X)$ on X would be expected to be large and the multiple correlation $R_{(W+X)\cdot X\sqrt{X}}$ would be expected to be large.

Equation (4) is plotted in Figure 1. The interval from calving to conception increases markedly as the service interval increases, as would be expected. After a service interval of 49 days, there is an increase in the interval from calving to conception of almost a day for each day the service interval increases. The graph of Equation (4) clearly indicates how prolonging the service interval increases the interval from calving to conception.

The regression of $(W + X)$ on X, $\sqrt{X}$, and Z was derived and is Equation (5)

$$\widehat{W+X} = 88.51 + 1.55\ (\pm 0.24)\,X - 12.88\,(\pm 4.50)\,\sqrt{X} + 0.09\,(\pm 0.05)\,Z$$
$$R_{(W+X)\cdot X\sqrt{X}Z} = 0.42 \qquad \text{D.F.} = 799 \qquad (5)$$

If Equation (5) is graphed for $Z = 204$, the resulting curve coincides almost exactly with that of Equation (4) in Figure 1. The service interval must exceed 160 days for the difference between corresponding regression values to exceed one day.

The level of butterfat production. From the correlations, means, and standard deviations in Tables 3 and 4, the regression of the adjusted butterfat production (in kilograms) on service interval (X) and $\sqrt{X}$ was derived and is Equation (6).

$$\hat{Z} = 245.26 + 0.69\ (\pm 0.18)\ X - 10.90 \pm (3.46)\ \sqrt{X} \qquad (6)$$
$$R_{Z\cdot X\sqrt{X}} = 0.137 \qquad \text{D.F.} = 800$$

Equation (6) is plotted in Figure 1 and, as is evident from Figure 1, the derivative of $\hat{Z}$ with respect to X gives a minimum butterfat production for $X = 63$. As the service interval increases from 63 to 169 days, there is a general increase in the level of butterfat production. As the service interval increases from 36 to 63 days there is a small, but probably not real, decrease in butterfat production. For service intervals from 49 to 90 days there is little difference in the levels of associated butterfat production.

The increase in the level of butterfat production associated with an increase in the length of the service interval could be brought about in several ways: (1) There could be a tendency to breed low-producing cows earlier after calving than high-producing cows. (2) High-producing cows may tend to show estrus at a larger interval postpartum than low-producing cows and, thus, drift into the classes with longer service intervals. (3) With the longer service intervals, conception occurs later after calving; thus, there is less interference of pregnancy with production. The first possible cause is not likely to be of importance, since the management practices were standard for cows at the testing stations. Clapp

TABLE 5

Tests of significance of the within-sire variance of the interval from first service to conception

Source	D.F.	M.S.
Service interval $(R^2_{W\cdot X\sqrt{X}Z} - r^2_{WZ})\ S_W 2$	2	9,991.8**
Butterfat production $(R^2_{W\cdot X\sqrt{X}} - R^2_{W\cdot X\sqrt{X}})\ S_W 2$	1	7,895.7*
Error $(1 - R^2_{W\cdot X\sqrt{X}Z})\ S_W 2$	799	1,978.8

** Significant at the 0.01 level of probability.
* Significant at the 0.05 level of probability.

(8) has presented information, though not conclusive, that the second cause may be real. Gaines (13), on the other hand, has presented data which indicate that the first full month's milk yield and the service period (the interval from calving to conception) are independent; thus, the second cause is not likely. It is probable that the third possibility is the real cause of the association.

Services per conception. Previous studies (6, 11, 12, 19, 21, 23, 24, 25) have dealt with the number of services per conception in relation to the length of the srvice interval. In the present data, the regression of services per conception on service interval, as shown in Equation (7) and in Table 6, was not significant.

$$\hat{Y} = 1.67 - 0.005\ (\pm 0.007)\ X + 0.03\ (\pm 0.14)\ \sqrt{X} + 0.0028\ (\pm 0.0014)\ Z$$
$$R_{Y\cdot X\sqrt{X}} = 0.084 \qquad \text{D.F.} = 799 \qquad (7)$$

If Equation (7) is plotted for $Z = 204$ kg., the resulting graph shows that there is a decrease in services per conception as the service interval increases; thus agreeing with the results in (6, 11, 12, 19, 21, 23, 24, 25).

Since most of the sum of squares of services per conception associated with service interval was associated with the linear component, the regression of services per conception on the first power of service interval and butterfat production was derived, and the two partial regression coefficients $b_{YX\cdot Z} = -0.0034 \pm 0.0023$ and $b_{YZ\cdot X} = 0.0028 \pm 0.0014$ were found. With 800 degrees of freedom, the partial regression -0.0034 is significant $(0.05 < P < 0.10)$. This result again substantiates previous conclusions that there is a general decrease in the services required per conception as the service interval increases.

TABLE 6

Tests of significance of the within-sire variances of services per conception associated with butterfat production and service interval

Source	D.F.	M.S.
Butterfat production $(R^2_{Y\cdot X\sqrt{X}Z} - R^2_{Y\cdot X\sqrt{X}})\ (S_Y 2)$	1	7.52*
Service interval $(R^2_{Y\cdot X\sqrt{X}Z} - r^2_{YZ})\ (S_Y 2)$	2	2.18
Error $(1 - R^2_{Y\cdot X\sqrt{X}Z})\ (S_Y 2)$	799	1.93

* Significant at the 0.05 level of probability.

TABLE 7

Tests of significance of the within-sire variance of total number of services associated with service interval and butterfat production

Source	D.F.	M.S.
Service interval ($R^2_{Y'\cdot XZ}$) $S_{Y'}2 - r^2_{YZ} S_{Y'}2$	1	12.269*
Butterfat production ($R^2_{Y'\cdot XZ}$) $S_Y 2 - r^2_{YX} S_{Y'}2$	1	9.875*
Error $(1 - R^2_{Y\cdot XZ})$ $S_Y 2$	849	2.32

* Significant at the 0.05 level of probability.

The partial regression of services per conception on butterfat production of 0.0028 is significant ($0.01 < P < 0.05$) and suggests that higher-producing cows require more services per conception. This relationship is probably brought about by the fact that cows requiring more services per conception are not pregnant as soon after calving and, thus, that their current production is less affected by pregnancy than is that of cows requiring fewer services per conception.

To provide evidence on this point, the regression of services per conception *(Y)* on service interval *(X)*, the interval from first service to conception *(W)*, and the level of butterfat production *(Z)* was derived and is Equation (8).

$$\hat{Y} = 1.19 - 0.0007 \pm (0.001)\, X + 0.0267\ (\pm 0.0005)\, W - 0.00004 \\ (\pm 0.0007) Z. \qquad (8)$$

The partial regression $b_{YZ\cdot XW} = -0.00004$ is not significant and is practically zero. Thus, it appears that there is no real biological basis for concluding that high-producing cows are less fertile than low-producing cows. This is in agreement with the conclusions of (2, 4, 10, 13) and in disagreement with the findings of (1, 3, 18, 20, 22). The relationships reported in (1, 3, 18, 20, 22) are, possibly, confounded with other factors.

All services. By studying services per conception, one includes only those cows that conceived. This, in effect, truncates the data to include only fertile cows. Equation (9) is the regression of all services *(Y')*, whether or not the cows conceived, on service interval *(X)* and butterfat production *(Z)*.

$$\hat{Y}' = 2.13 - 0.0056\ (\pm .0024)\, X + 0.0031\ (\pm .0015)\, Z \qquad (9)$$

$$R_{Y'\cdot XZ} = .104 \qquad \text{D.F.} = 849$$

A prediction equation including $\sqrt{X}$ was first derived, but the partial regression of *(Y')* on $\sqrt{X}$ was extremely small and not significant; consequently, it was not included and Equation (9) was used. The regression coefficients in Equation (9) are based on 849 degrees of freedom and are significant at the 0.05 level of probability. The variances independently accounted for by service interval and butterfat production are shown in Table 7. In spite of their significance, the two together account for only 1.1% of the within-sire variance of number of services. For a constant butterfat production, the number of services goes up 0.56 of a service for each 100 days the service interval increases.

DISCUSSION OF RESULTS

The results indicate that the number of services per conception (Y) decreases a small, though not significant, amount for each day the service interval (X) increases, the equation for a constant level of butterfat production $(\overline{Z})$ being $\hat{Y} = 2.38 - .0034\,X$. The results also indicate that the interval from first service to conception (W) decreases at a decreasing rate as the service interval (X) increases to 127 days. With a service interval of 127 days, the interval from first service to conception reaches a minimum of 32.8 days.

Both a small number of services per conception and a short interval from first service to conception are important items and are to be desired by a dairyman. However, the attainment of a minimum number of services per conception and a minimum interval from first service to conception are not the only items to be considered in recommending an optimum interval from calving to first service. The interval from calving to conception should definitely be considered, as it and the length of the gestation period determine the length of the calving interval. Most of the variation in calving interval is brought about by variation in the interval from calving to conception, because the variation in the length of the gestation period is relatively small. From results in (9) and Tables 3 and 4 of this study, it appears that about 99% of the variation in calving interval is caused by variation in the interval from calving to conception.

The important thing to remember concerning the service interval, services per conception, the interval from first service to conception, and the interval from calving to conception is: that variation in service interval accounts for approximately 1% of the variation in services per conception and the interval from first service to conception; whereas, variation in the service interval accounts for approximately 17% of the variation in the interval from calving to conception and, thus, of calving interval. Thus, it would seem that the length of the calving interval desired would be the primary consideration in recommending a short or long service interval.

Equation (10) expresses the relationship between the interval from calving to conception and service interval for the mean Z and, as mentioned previously, almost coincides with that plotted in Figure 1.

$$(\hat{W} + X) = 107.13 + 1.55^{**}\,X - 12.88^{**}\,\sqrt{X} \qquad (10)$$

If one assumes that the length of the service interval has little or no effect on the length of the gestation period, then the relationship between service interval and calving interval is expressed by adding the gestation period to Equation (10). Assuming a gestation period of 280 days, the calving interval for a mean Z may be expressed as shown in Equation (11).

$$\text{C.I.} = 387.13 + 1.55\,X - 12.88\,\sqrt{X} \qquad (11)$$

By taking the partial derivative of C.I. in Equation (11) with respect to (X), setting the derivative equal to 0, and solving for X, it is found that the average minimum calving interval occurs when the average service interval (X) is

17.2 days. This value of X is outside the range of service intervals in these data and is one that is unrealistic. For service intervals of 36, 49, 64, 81, and 100 days, which are more nearly within the range of the present data, one would expect calving intervals of 366, 373, 383, 397, and 414 days, respectively.

If the expected butterfat production values in Figure 1 for Equation (6) are divided by the corresponding expected calving intervals derived above, the production per calving interval day decreases as the calving interval increases. For example, for service intervals of 36, 49, 64, 81, and 100 days, the expected calving intervals are 366, 373, 383, 397, and 414 days, the expected butterfat production is 205, 203, 202, 203, and 205 kg., and the expected production per calving interval day is 0.56, 0.54, 0.53, 0.51, and 0.50 kg., respectively. The present study agrees with the results of Gaines (14) in regard to current calving interval. Gaines (14) obtained a correlation of -0.134 ± 0.018 between current calving interval and current production per calving interval day, and a correlation of 0.142 ± 0.018 between the previous calving interval and current production per calving interval day. The correlation, 0.142, between previous calving interval and current production per calving interval day would be partially confounded with age effects, since cows having longer previous calving intervals are likely to be older at calving; thus, the correlation of 0.142 is probably too large. Consequently, the conclusion of Gaines (14) that what is gained in the current lactation by having a short calving interval is lost in the next lactation is questionable. Chapman and Casida (7) concluded that there was no marked reduction in production per calving interval day in the second of two consecutive short calving intervals. Johansson and Hansson (17) concluded that the optimum length of the first calving interval would be about 410 to 430 days, and about 400 days for the second. For the third and, probably, later lactations any length between 310 and 430 days gives about the same yield per calving interval day. On a basis of these references, calving intervals of 12 to 13 mo. seem to be desirable.

The real solution for the optimum length of calving interval is, within biological limits, an economic one and depends on the relative economic values of calving intervals of varying lengths, including yield per calving interval day, seasonal variations in the price of milk, number of calves born, and varying number of services required per conception. Under present economic conditions, with the seasonal variations in milk prices, an average calving interval of approximately 12 mo. would seem to be desirable.

It should be remembered that at present under practical conditions man's control over calving interval is limited to that period between the time when the cow first comes into heat after calving and the time when the first service is made. There is more freedom to make this period long than there is to make it short. Artificial breeding organizations would be interested in a low number of services per conception, as this would be less expensive to the organizations where second and third services are given free of charge, when the first or first and second services fail to get the cow in calf. Thus, it is likely that artificial breeding organizations would recommend a relatively long service interval. On the

other hand, a shorter service interval might result in more first services per cow, since the calving intervals would be shorter, thus producing more income for artificial breeding organizations.

If one reduces the number of services required per conception from that found in these data, it would probably be possible to allow a longer service interval and still maintain a 365-day or shorter calving interval. In the present data, the linear regression of the interval from first service to conception *(W)* on services per conception *(Y)* is 27.64; thus, if the average number of services per conception was reduced from 2.09, as found in these data, to 1.67, which would correspond to a 60% conception rate, the interval from first service to conception would be reduced by approximately 11.6 days. Assuming that this effect is the same for service intervals of different lengths, one could allow an average service interval of from 45 to 60 days and still maintain a 365-day calving interval. If one reduced the length of normal gestations by 15 to 30 days, it would be possible to allow a 15- to 30-day longer service interval and still maintain a 365-day calving interval. Although it has not been conclusively established that the gestation period can be shortened without associated deleterious effects, work by DeFries *et al.* (9) indicates that selection for a shorter gestation is feasible.

Other workers (1, 3, 18, 20, 22) have presented data which indicate that high-producing cows require a considerably increased number of services per conception. These data could be biased, in that there would be a tendency to be more patient with high-producing cows and allow them more chance to conceive than would be the case with low-producing cows. It is also possible that service sire differences are confounded with production levels. In addition, it is probable that those cows requiring more services have a longer period from calving to conception and, thus, less effect of pregnancy on lactations.

By utilizing the present data, the partial regression of services per conception on butterfat production for a constant service interval and a constant interval from first service to conception is -0.00004 ± 0.0007. From the correlations, standard deviations, and means in Tables 3 and 4, the partial regressions $b_{Y(W+X)\cdot Z} = 0.022$ and $b_{YZ\cdot(W+X)} = -0.0007$ can be derived. This suggests that the association between the level of butterfat production and services per conception is largely a result of the corresponding relationship between the interval from calving to conception and butterfat production; thus, there is no evidence in these data to suggest that the biological relationship between services per conception and the level of butterfat production is different from zero.

Artificial breeding associations would probably be more interested in total number of services as related to service interval rather than services per conception because, at the time of first service, no one really knows which cows will conceive and which will not. As shown in Equation (5), 0.56 less services can be expected for each 100 days of increase in the length of the service interval.

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DEVELOPMENT OF RUMEN FUNCTION IN THE DAIRY CALF. II. EFFECT OF DIET UPON CHARACTERISTICS OF THE RUMEN FLORA AND FAUNA OF YOUNG CALVES[1,2]

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SUMMARY

Twenty-four male calves were placed, at an average age of 3.5 days, on diets representing liberal and limited milk-feeding programs. The calves on limited milk were further divided into those receiving aureomycin in the concentrate mix and those not receiving it. In addition, a group of calves received only milk for 8 of the 12 wk. of the experiment. Rumen samples were taken weekly and analyzed for color, odor, consistency, total bacteria, aerobic bacteria, morphologically characteristic organisms, protozoa, the levels of niacin, biotin, and riboflavin, cellulose digestion, and the amounts of the lower fatty acids (C_2–C_4). For calves fed solid feeds, the general pattern of rumen function development seemed to be a sharp fall in the aerobic bacteria within the first 3 wk., an increase in the total numbers of bacteria, and the establishment of protozoa. The rumen acids were at adult levels by 6 wk. of age; whereas, the protozoal and bacterial numbers approached this point a little later. The establishment of adult-type organisms and cellulose digestion were slowest to develop and were hindered by aureomycin and liberal milk feeding. Biotin, niacin, and riboflavin levels increased up to 7 wk. of age, with an indication of synthesis early in life.

Much effort has been given to exploring the possibility of fostering an adult-like flora and fauna in the rumens of very young calves by the use of cud inoculations and/or high-roughage diets (4, 5, 8, 9, 10, 17, 18). To properly evaluate such programs, it seemed necessary to describe the establishment of the rumen organisms in calves raised under conditions of management and feeding on the farm.

As in the more recent publications on this subject (4, 5, 6, 8), it was felt that more criteria than the morphologically characteristic organisms would be needed. Therefore, it was decided to count the protozoa and bacteria and to assay for the ability to digest cellulose and the production of vitamins and fatty acids. In addition, the rumen characteristics of the adult animals in the herd were established (12), to serve as a base line.

METHODS

All samples were collected by stomach tube 6 hr. after feeding. After noting color, odor, and consistency, the rumen contents were strained through cheese cloth and the solid portion used for total bacterial counts, identification of

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characteristic organisms, aerobic plate counts, and the levels of niacin, biotin, and riboflavin. The liquid portion was used for protozoa counts, assay of fatty acids, and to test cellulose-digesting ability of the flora.

The fraction of the rumen contents, liquid or solid, used for a particular test, was chosen on the basis of where the numbers or concentrations could reasonably be expected to be greatest. Since the cellulolytic bacteria are intimately associated with the rumen solids, the solid portion was used for the identification and counting of bacteria. The bacteria and plant material are the sources of the vitamins; therefore, the solid portion was also used for the assay of the B-vitamins. The free swimming protozoa and the end-products of bacterial metabolism, the fatty acids, are found in the liquid portion, so this was used for their enumeration. In instances where no solids were obtained, the liquid was used instead.

Standard bacteriological techniques, described at length in a previous publication (12), were used in making slides for bacterial counts and for the plate counts of aerobic organisms. For identification of characteristic organisms, the system used by Rusoff *et al.* (19), to which was added a G^- spiral-like organism, 4 to 18 μ in length and 0.5 μ or less in width, was employed. The ability of the rumen organisms to produce gas from Solka-Floc was used as a criterion of cellulose digestion, as was a drop-weight technique developed by O'Neil *et al.* (16). Protozoa were counted by means of a Spencer Brightline hemocytometer (12). For the rumen acids, the method of Neish (15), as modified by Bauman (2), was employed. The method of Genghof *et al.* (7) was used to analyze for biotin, and the procedures of Bacharach and Cuthbertson (1) for riboflavin and niacin. The data were analyzed by means of the analysis of variance (20).

EXPERIMENTAL PROCEDURE

A total of 24 male calves—18 Holsteins, three Brown Swiss, two Guernseys, and one Ayrshire—were used for this study. The calves averaged 3.5 days of age and had not consumed solid foods at the start of the experiment. The experiment started in September of 1952 and lasted through May of 1953. Six animals comprised a group, though all were not on trial at the same time. Each animal was under observation for a period of 12 wk. As they were born in the herd, calves were assigned in rotation to the experimental groups.

The objective was to compare the development of the rumen flora and fauna when milk was the only nutrient, when calves received liberal amounts of milk along with hay and grain, and when animals were on a limited milk-feeding program, with and without antibiotics.

The group of calves designated as normal received milk for the entire experimental period at the rate of 10% of the body weight, with a maximum of 10 lb. per day. A 14% crude protein concentrate composed of 700 lb. each of corn and oats, 400 lb. of bran, 200 lb. of linseed oil meal, and 20 lb. each of bone meal and salt was fed free-choice, with a maximum offering of 3 lb. per day. Water and hay were supplied ad lib. In this and all other cases where hay was fed, a clover-timothy mixed hay of fair quality was used.

Two groups were placed on a limited milk program comparable to the system commonly used for raising calves for herd replacements. They received milk at the rate of 10% of the body weight during the first 3 wk. After this period, the milk was gradually withdrawn from the diet and no milk was offered after the end of the 7th wk.

Concentrate consisting of 480 lb. corn, 500 lb. linseed oil meal, 400 lb. corn gluten meal, 200 lb. each of soybean oil meal, oats, and wheat bran, and 20 lb. salt, approximately 25% crude protein, was fed free-choice with a limit of 3 lb. per day. One of these groups was designated as nonaureo and received the grain ration without additions. For the other group, called aureo, 40 g. of crystalline aureomycin-hydrochloride were mixed into each ton of concentrate. Water and hay were fed ad lib.

The fourth group of calves, the milk group, for the first 8 wk. received only milk, at the rate of 12% of the body weight per day. At the start of the 9th wk., the 14% crude protein concentrate and hay were offered. The milk was then rapidly reduced, so that no milk was offered by the end of the 11th wk.

All calves were kept in elevated mesh-floored tie stalls for the entire 12-wk. experimental period. They were housed in the same barn as adult cattle, but care was exercised to prevent contact or contamination. They were fed at 7 A.M. and 4 P.M. each day. Rumen samples were collected by stomach tube once a week at 1 P.M.

RESULTS

The rumen samples indicated that the calves which had the opportunity consumed appreciable amounts of solid feeds as early as 2 wk. of age. The rumen contents closely resembled those of adult cattle in color, odor, and consistency by 3 and 4 wk. of age, for calves on the limited and liberal milk feeding programs, respectively. The calves receiving only milk manifested a craving for dry feed at 2 wk. of age, and at 8 wk., when first offered hay and concentrate, avidly consumed from 3 to 5 lb. of hay per day.

With time, a definite increase in the number of varieties of microorganisms in the rumen was noted (Table 1). In the milk group, only a few types were present until the feeding of hay and grain began. When supplied with solid feeds it took but 3 wk. to produce a flora as diversified as that of the adult (12). Few differences were observed between normal and nonaureo groups, which represented liberal and limited milk-feeding programs, respectively. A flora as varied as that of the adult animals in this herd had developed in these calves by the 8th wk. The aureo group consistently showed a relative lack of organisms classified as Hay IIa and Spirals.

Counts of the rumen bacteria indicated that the number of organisms increased as the calves grew older (Figure 1). For calves receiving only milk, the bacterial count remained low until hay and concentrate were supplied. The numbers then increased markedly and at the 10th wk., 2 wk. after being offered solid foods, the bacterial count was equal to that of the other groups. For reference purposes, adult cattle of this herd had been observed (12) to have about 66 billion organisms per gram of rumen content.

TABLE 1

Influence of diet on the development of characteristic organisms in the rumen of the calf[a]

Week	Milk						Normal						Aureo						Nonaureo					
	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral
0	0	1	0	6	2	0	0	0	0	6	4	0	0	0	0	5	4	2	0	0	2	5	4	0
1	0	0	1	6	1	1	0	0	0	6	3	5	1	1	0	6	4	2	1	0	2	6	2	3
2	0	0	0	6	0	0	0	0	0	6	5	4	2	2	2	6	6	1	3	1	2	6	6	4
3	0	0	0	6	3	1	1	1	3	6	6	5	3	3	3	6	6	0	2	0	3	5	6	4
4	0	0	1	6	2	0	2	1	2	6	6	5	4	3	2	6	5	2	2	0	3	6	6	6
5	0	0	2	6	4	0	3	1	5	6	6	5	5	4	0	6	5	1	3	2	4	6	6	4
6	0	0	1	6	4	0	4	1	4	6	6	5	6	3	3	6	5	2	5	2	3	5	6	4
7	0	0	1	6	4	0	4	2	3	6	6	6	4	1	1	6	6	0	5	2	3	6	6	4
8	0	0	1	6	6	0	5	3	2	5	6	5	5	0	3	6	6	0	5	4	3	6	6	6
9	3	1	2	6	6	4[b]	4	5	2	5	6	6	5	0	4	6	6	1	6	4	5	6	6	6
10	3	1	4	6	6	5	5	2	1	6	6	6	6	0	3	6	6	1	6	4	1	6	6	6
11	4	2	4	6	6	6	5	3	0	6	6	4	6	0	0	6	6	1	6	4	4	6	6	6
12	5	0	6	6	6	6	5	3	0	6	6	6	6	0	1	6	6	2	6	3	3	6	6	6

[a] A group consisted of six animals. Each figure in the table indicates the number of animals showing the organism.

[b] Hay and concentrate feeding initiated; milk reduced.

The numbers of aerobic bacteria showed marked changes during the course of the experiment (Figure 1). Most striking was the presence of approximately 240 million per gram of rumen contents at the first sampling. A sharp drop in numbers occurred during the 1st wk. of the experiment. In animals receiving solid foods, this new level tended to be maintained for another week and then decreased again to about three million bacteria per gram of rumen solids. This was lower than the ten million aerobes per gram of rumen solids noted for adult cattle. No differences were observed between normal and nonaureo groups; but after the 4th wk. the group fed aureomycin consistently showed about half the number of aerobic bacteria that the nonaureo group of calves showed.

Those animals maintained solely on milk showed very high levels of aerobic bacteria in the rumen contents. The lack of fibrous solid material, necessitating in some cases the use of liquid ingesta for bacterial counts, may have been a contributing factor to the differences in aerobe numbers. When supplied with solid foods, these animals followed the pattern of the other groups.

As seen in Figure 1, protozoa were established within the rumen during the 1st wk. on experiment. For calves receiving aureomycin, the numbers increased rapidly and equaled the adult level (477,000 per milliliter) at about 5 wk. of age. The nonaureo group exhibited a 2-wk. lag when compared to the aureo animals; however, both groups reached a similar peak at 11 wk. of age. The normal calves, receiving milk throughout the experiment, showed a slow development of the fauna and did not attain the numbers seen in adult cattle until 9 wk., and never approached the values for the nonaureo or aureo calves.

Most striking was the relative lack of a protozoan population in calves receiving only milk. These animals maintained a low count during the 9 wk. when only milk was fed, and for about 3 wk. after hay and grain feeding was begun. At 12 wk., the protozoa began to increase in number, a situation similar

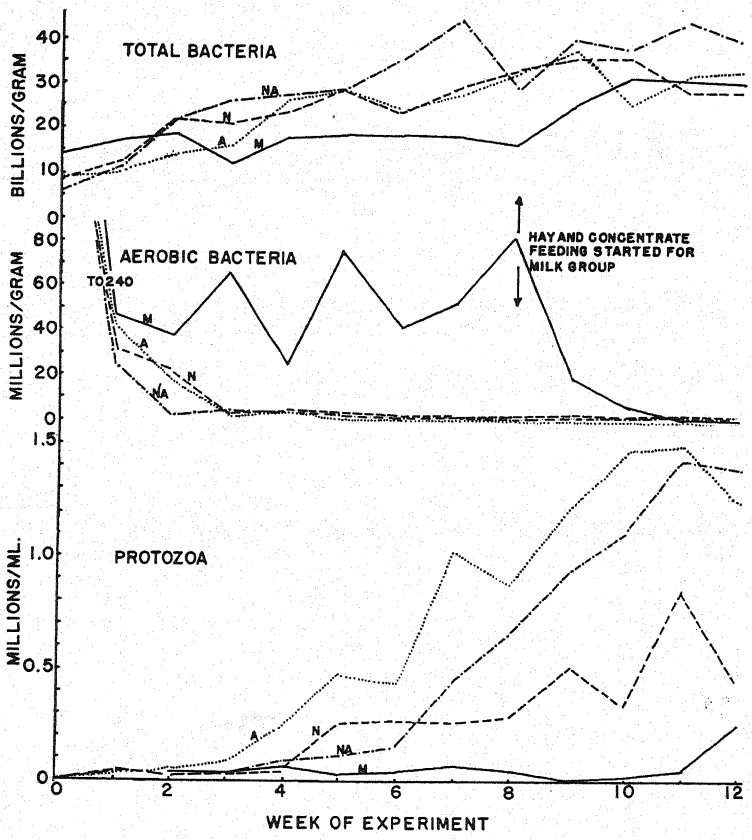


FIG. 1. The numbers of all bacteria, aerobic bacteria, and protozoa found in the rumen contents of calves raised under different regimes. For this figure, N indicates the normal group, M the milk, A the aureomycin, and NA the nonaureomycin group.

to that for 4-wk.-old calves fed hay and grain from the start. Thus, hay and concentrate seem to be necessary for the normal proliferation of the protozoan population.

Differences in the characteristics of the protozoan population were followed, as well. Only small ($< 5\ \mu$ in length) protozoans were present in the rumen of animals receiving only milk. When solid feeds were provided, the large ($> 5\ \mu$ in length) protozoa appeared. The aureomycin-fed calves exhibited the large protozoa in the rumen contents in all cases after 6 wk. on experiment; this was true for the nonaureo group at 11 wk. In the normal group, five of the six calves showed the presence of large protozoa at the 12th experimental week. In all calves, only a few heterotrichous types of protozoa (large) developed. Two calves receiving cud-inoculum developed a protozoan population which contained numerous varieties of both holotrichous and heterotrichous species.

Attempts were made to estimate the cellulolytic activity of the rumen flora. The criteria were the time to digest a cotton thread and the production of gas

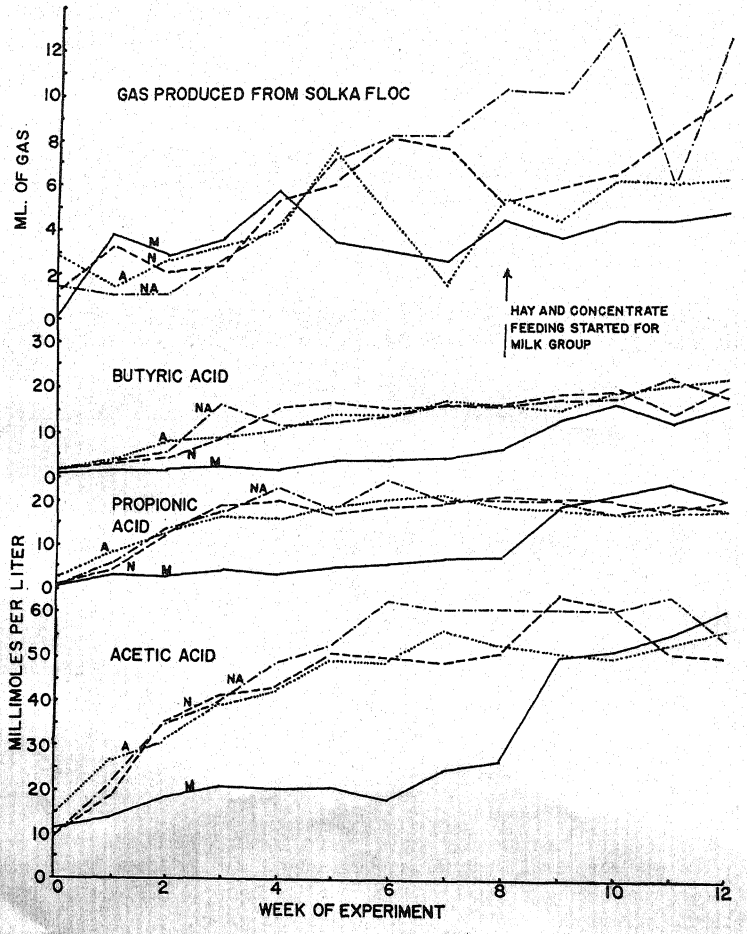


FIG. 2. The in vitro digestion of cellulose and the fatty acid concentration of the rumen contents of calves kept under different dietary conditions. For this figure, N indicates the normal group, M the milk, A the aureomycin, and NA the nonaureomycin group.

as a result of bacterial attack upon Solka-Floc. The data for the latter technique are presented in Figure 2.

In all groups, a gradual increase in cellulolytic activity was noted up to 4 wk. of age. At this time the animals fed only milk plateaued and never reached levels comparable to the other dietary groups. The aureo calves leveled off at 5 wk., apparently due to the inhibitory effect of aureomycin, at a value about one-half that for adult cattle (9.4 ml. of gas) and significantly lower ($P < 0.05$) than the nonaureo calves at Weeks 9, 10, and 12. The nonaureo and normal groups approached the adult level of activity at 6 wk. of age.

The test employing the breakage of a cotton thread indicated that the nonaureo calves were equivalent to adult cattle at 9 wk. of age. Aureomycin and milk feeding seemed to be inhibitory; however, it must be noted that even the rumen contents of the milk-fed group were capable of digesting cellulose.

Weekly analyses of the rumen levels of various acids were made throughout the experiment; the data are shown in Figure 2. Most striking are the low levels of butyric, propionic, and acetic acids found in the rumen contents of the milk-fed group during the period of milk feeding. This group was statistically different ($P < 0.05$) from all the other groups from Weeks 2 through 9. With the start of hay and concentrate feeding, the levels of the acids rose rapidly to become indistinguishable from those of the other groups. It should be noted that the rise at the 9th wk. was much sharper than the rises shown in the early weeks by those animals receiving hay and concentrate from the start. The difference possibly can be attributed to the comparatively large amounts of hay and concentrate consumed by the milk-fed calves when their diet was changed.

For the other dietary groups, few differences were noted. In general, these groups showed levels of butyric acid comparable to the adult (14.5 m*M*/liter) at 4 wk. For propionic acid, the adult value (18.1 m*M*/liter) was reached at the 3rd wk.; the acetic acid values took longer to plateau, but were at levels similar to those for adult cattle (63 m*M*/liter) at 6 or 7 wk. of age.

The formic acid content of the rumen material showed little variation, the values hovering around the levels established for adult animals (1.3 m*M*/liter). The milk-fed calves remained somewhat below this value ($\cong$ 0.8 m*M*/liter) and at the 2nd, 4th, and 6th wk. were found ($P < 0.05$) to be different from all other groups.

The succinic-lactic acid in the rumen appeared to vary widely around the level found for adult cattle (5.24 m*M*/liter) and few differences were observed. A tendency was seen for this group of acids to be high (10 to 30 m*M*/liter) during the early weeks and then to fall off. Only the nonaureo group showed that the values for Weeks 1 and 2 were significantly higher ($P < 0.05$) than for Weeks 3, 6, and thereafter.

Expressing the data of each acid as per cent of the total of the rumen acids titrated, it was noted that for the milk-fed animals the per cent of acid was significantly lower ($P < 0.05$) at Weeks 7 and 8 for butyric and at Weeks 3 through 7 for propionic, than for calves of the other groups. Conversely, the proportions were greater ($P < 0.05$) for acetic at Weeks 2, 3, 4, and 6 and for formic at Weeks 5, 6, and 7. For the succinic-lactic acids, no real differences were seen between groups; however, this fraction formed a large portion of the rumen acids during the first sampling period and was lower ($P < 0.05$) after 3 wk. In the first sampling period, the succinic-lactic fraction averaged 41% of all the acids titrated; whereas, at 12 wk. it represented but 5.3%.

The levels of some of the B-vitamins in the rumen solids were determined and are shown in Figure 3. For biotin, no significant differences could be detected, though the aureomycin-fed calves consistently averaged higher than other groups. The aureo group showed a trend for the levels of biotin in the rumen to increase during the experimental period. Statistically, the levels at Weeks 1 and 2 were lower ($P < 0.05$) than those for Weeks 7 and thereafter.

The data for niacin (Figure 3) showed no differences between groups, though the aureomycin-fed calves, during Weeks 5 through 11, consistently averaged

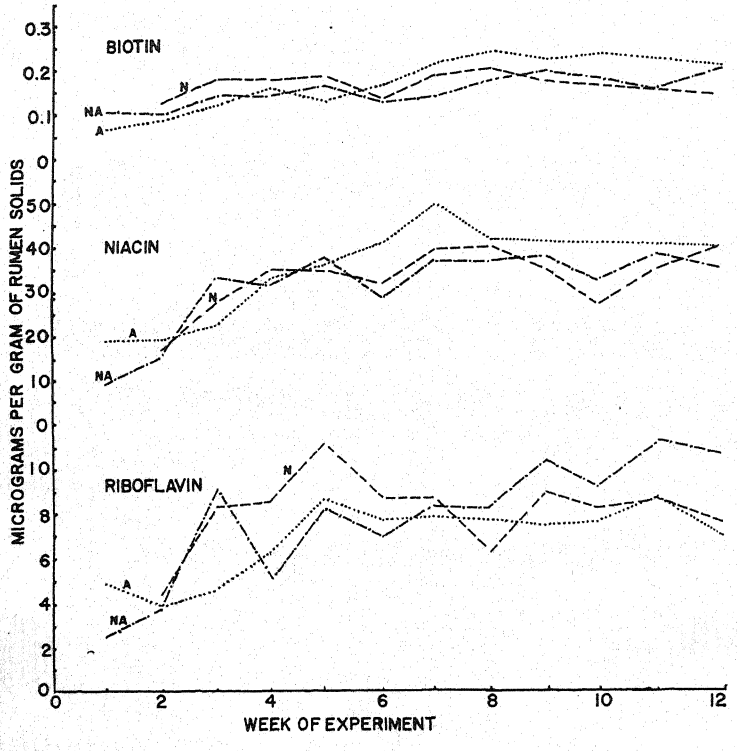


FIG. 3. The concentration of some B-vitamins in the rumen fluid of calves raised under various feeding systems. For this figure, N indicates the normal group, A the aureomycin, and NA the nonaureomycin group.

higher. Only in this group could an indication of an increase with age be shown. The value for Week 1 was lower ($P < 0.05$) than the values for weeks thereafter; Week 2 differed from Weeks 5 and thereafter; and Week 3 differed from weeks thereafter.

For riboflavin (Figure 3), no differences existed between groups. Some suggestion of an increase in the levels of riboflavin in the contents could be seen; however, this could be shown statistically for the nonaureo group only. For these animals, Week 1 differed ($P < 0.05$) from Weeks 9 and thereafter; Week 2 differed from Weeks 9, 11, and 12.

For comparative purposes, the concentrates fed were found to contain 0, 4.5, and 0.013 γ/g of dry matter of riboflavin, niacin, and biotin, respectively. The hay contained 4.8, 12.2, and 0.010 γ/g of dry matter of riboflavin, niacin, and biotin, respectively. Milk contained 0.96, 2.89, and 0.025 γ/g of riboflavin, niacin, and biotin, respectively. The solids obtained from the rumen contained ten times as much biotin at the 2nd wk. of age as was present in the concentrate or hay. The niacin levels were two to three times those of the roughage, and for riboflavin were about one and one-half times richer. Since the rumen liquid was also certain to contain some of these vitamins, this is evidence of very early synthesis of these vitamins in the rumen of the calf.

DISCUSSION

These experiments indicate that functional development of the rumen, similar to that seen in mature animals, can be attained at very young ages in dairy calves by using standard feeding practices. Few differences attributable to alterations in the feeding program could be seen among calves receiving hay and concentrate.

Using the nonaureo group as being indicative of the rumen function development of calves raised on a limited milk-feeding program without antibiotics, it was seen that the rumen acids had reached adult levels, the vitamin content of the rumen solids had plateaued, and cellulose digestion (gas-production method) could not be distinguished from the adult by the 6th wk. of the experiment. The numbers and types of bacteria and protozoa took longer to develop, but resembled the adult at the 8th wk.

This group of calves thus closely resembled the cud-inoculated calves of Bryant and Small (4) and calves fed high-roughage pellets by Conrad *et al.* (6). The most striking difference was the development of a protozoan population [the uninoculated calves of Conrad *et al.* (6) failed to have this development]. The population was not as diverse as that of two cud-inoculated calves or the calves of Bryant and Small (4). It is evident that some contamination from the adult animals did occur, even though there was no direct contact or use of common utensils. This indicates that when calves are kept in the proximity of mature animals, special measures to provide them with cud-inoculum need not be taken. This is supported by the finding of no significant differences in the performance of uninoculated calves on high-roughage diets, when compared to those receiving rumen inoculum (8).

A liberal milk-feeding program, as represented by the normal group, seemed to delay the acquisition of some adult characteristics. In most cases, adult values were reached from 1 to 2 wk. later than in the nonaureo calves. By 9 wk., the rumen function was equal to that of adult cattle in all respects except the digestion of cellulose. By the 12th wk., even this goal was attained. It is to be noted that the nonaureo and normal groups acted alike up to 6 wk. of age, then differed. The rapid withdrawal of milk from the diet of the nonaureo group seemed to accelerate the maturation of the rumen function. Milk has been noted to have a depressing effect upon the size of the rumen protozoal population (13) and may explain the marked increase in the numbers noted in the nonaureo group when milk was withdrawn; whereas, a relatively slow increase took place in the normal calves. Somewhat the same picture was presented for the cellulose-digesting ability of the rumen liquor.

Aureomycin had an enhancing effect, in that the protozoal population seemed to be fostered, which agrees with the data of Mann *et al.* (14), and the biotin and niacin levels of the rumen contents were higher than observed for the other groups. However, the aureomycin did have an inhibitory effect on the microorganisms of the rumen. Of the indicator organisms, the Hay IIa, confirming Hibbs and Conrad (9), and the Spiral, were lacking. Also, it was noted that the rumen contained fewer aerobic bacteria than did the other groups. From this, it

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can then be surmised that other, less distinctive types of organisms were affected, as well. This probably contributed to the lessened ability to digest cellulose, an observation also noted by some others (3, 6, 11).

The calves fed only milk showed a rumen function that was markedly immature when compared to the other animals; however, some cellulolytic activity was present (5). The supplying of hay and grain brought about the immediate appearance of new organisms which were more characteristic of adult rumen contents; the amounts and percentages of the rumen acids also came to resemble those of the other groups more closely. The results obtained with this group, and the less dramatic differences seen between the other groups, lead to the conclusion that the major factor affecting rumen function development was the diet. All animals on a diet containing hay and concentrate rapidly developed the same characteristics of rumen population as seen in the adult animals of this herd. Thus, it is evident that inoculation occurs as a natural event and that the fostering of such a population is mostly a problem of management of the substrate presented to the organisms, and that feeding practices now in use are successful in promoting early functional development.

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EFFECT OF ETHANOL ON RUMEN FERMENTATION[1]

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SUMMARY

Ethanol (800 ml., 47.5%) was administered via rumen fistula during the once-daily feeding of: (1) 14 lb. timothy hay plus 4 lb. of equal parts of ground corn and ground corncobs or (2) Ration 1 with 3% urea replacing corn or (3) 20 lb. alfalfa hay, to twin 1,100-lb. cows. Each ration was fed with and without ethanol to each cow for 14-day periods. Four per cent of ground ration was added to cheese cloth–strained rumen fluid and incubated for 3 hr. in vitro. Ethanol was not fermented in vitro but disappeared from the rumen with a half-time of 2 hr. Blood contained 75 mg.% ethanol 2 to 4 hr. after administration. Ethanol depressed rumen fill, gas production in vitro, and the utilization of radioactive sulfate for synthesis of amino acids and glutathione, but had little effect on the volatile fatty acid concentration in the rumen. Digestibility of crude protein and organic matter was slightly reduced by feeding ethanol with grain rations (1) and (2) to four dry nonfistulated cows.

Recent reports on the value of adding ethanol to rations for growing beef heifers (8), fattening cattle (9), growing dairy heifers (2), and lactating cows (1) have been inconclusive. Tsuda (11) has reported that ethanol is rapidly absorbed from the rumen. The present study was initiated to determine the effect of ethanol on rumen fermentation and to investigate its absorption from the rumen.

EXPERIMENTAL PROCEDURE

Twin 1,100-lb., dry Holstein cows, equipped with rumen fistulas, were fed the following ration once daily: 14 lb. of timothy hay plus 4 lb. of a grain mixture, consisting of equal parts of ground corn and ground corncobs plus salt and minerals (Ration 1). When 3% urea was added to this ration, the proportion of corn and corncobs was altered to maintain an equal TDN (Ration 2). Ration 3 consisted of 20 lb. of alfalfa hay. One twin received Ration 1 followed at 14-day intervals by Ration 1 plus ethanol, Ration 2 plus ethanol, and then Ration 2. Six months later, this same cow was fed Ration 3 plus ethanol for 21 days, followed by Ration 3 for 21 days. During these same 14- and 21-day periods, the other twin was fed successively Ration 1 plus ethanol, Ration 1, Ration 2, Ration 2 plus ethanol, Ration 3, and Ration 3 plus ethanol. Blood and rumen samples were taken 0, 2, and 4 hr. after feeding at the end of each period.

When Rations 1 and 2 were fed, the rumens were emptied of material on the last day of each period, 4 hr. after feeding. When ethanol was administered, 800 ml. of 47.5% alcohol was poured into the rumen fistula at the time of feeding and mixed with the contents. For the in vitro fermentations, a 3.5-hr. incubation period was used for the determination of gas production. Inoculum consisted of cheese cloth–strained rumen fluid. A 25-ml. portion was used for the ammonia

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and sulfate incorporation trials and a 20-ml. portion was used for the determination of gas production. The substrate level was 2 to 4 g. per 100 g. of inocula and consisted either of ground corn, of ground hay, or of combinations of the two. Where ammonium acetate was added, the level was 0.1 g. per 100-ml. inoculum. These fermentations were carried out in stoppered Erlenmeyer flasks in a 39° C. water bath.

Ammonia formation was determined in 36 flasks, of which 18 were controls and 18 contained 500 mg. % of ethanol. Three incubation times of 0, 1.5, and 3 hr. were used with each of three substrates: corn, corn plus ammonium acetate, and hay plus ammonium acetate. Two of the four flasks in each substrate-time subgroup contained inoculum from one twin; the two other flasks contained inoculum from the other twin. One of the two flasks in each set contained ethanol. Incorporation of inorganic sulfate was determined in 16 flasks. Two control flasks and two flasks with 500 mg. % ethanol were used for each of the twin cows when they received the hay ration. The entire trial was repeated when the hay ration plus ethanol was fed. Each flask contained 25 ml. inoculum, 0.25 g. corn, 0.25 g. hay, and 250 μc. of sulfur^{35}. Incubation time was 3 hr. Statistical significance was determined by analysis of variance.

The Keeney column (6) was used for the determination of volatile fatty acids, aeration for the determination of ammonia, aeration plus urease for the determination of urea, and a micro-diffusion method (7) for the determination of blood and rumen alcohol. The microbial amino acids and free glutathione were separated, identified, and assayed as described by Emery *et al.* (4).

Digestion and nitrogen balance trials were conducted with four successive ten-day preliminary and seven-day collection periods. In each period, four rations consisting of 12 lb. timothy hay plus 6 lb. of the corncob-corn mixture were fed alone, or with urea, or ethanol, or urea plus ethanol. The same four nonfistulated dry cows were used in all periods and were assigned to the rations at random. These rations were fed in equal portions, morning and evening. The ethanol also was fed twice daily by pouring it on the grain at feeding time.

RESULTS AND DISCUSSION

The mean concentration of blood alcohol 2 to 4 hr. after administration via the rumen fistula was 75 mg. %. The rumen concentrations 1, 2, and 4 hr. after administration of the ethanol were: 369 mg. %, 265 mg. %, and 145 mg. %, respectively. If these values are plotted on semilog paper and extrapolated back to the time of administration, and the average rumen volume as measured by emptying the rumen is taken into account, one finds that the maximum theoretical concentration of rumen ethanol was 500 mg. %. This alcohol concentration (500 mg. %) was then used for the in vitro fermentations when measuring ammonia metabolism and sulfate incorporation into amino acids. When gas production was determined, the ethanol naturally present in the inoculum as taken from the animal at about 2 hr. after feeding was sometimes used without further addition of alcohol. The concentration of rumen ammonia did not change with the administration of ethanol, although it was significantly affected by ration

TABLE 1
Ammonia nitrogen and fatty acids in rumen contents

	Rations					
	Corn-Corncobs-Timothy		Corn-Corncobs Timothy-Urea		Alfalfa hay	
	Control[a]	Ethanol[a]	Control[a]	Ethanol[a]	Control[a]	Ethanol[a]
Acetic Acid *(meq/l)*	56.8	63.8	67.5	66.3	99.9	98.4
Propionic Acid *(meq/l)*	14.3	16.6	18.6	16.3	28.0	28.7
Butyric Acid *(meq/l)*	8.2	11.2	9.7	9.2	15.6	12.7
Valeric Acid[b] *(meq/l)*	1.2	1.5	1.1	1.8	3.0	3.6
"Higher" acids *(meq/l)*	0.4	0.4	0.6	0.6	0.6	0.9
Total acids *(meq/l)*	80.9	93.5	97.5	94.2	147.1	144.3
Ammonium-N[c] *(mg. %)*	5.7	5.4	10.6	13.3	26.6	26.7

[a] Two samples per subgroup for acids, six for ammonia-N.
[b] P = about 0.3 for ethanol vs. control, combined rations.
[c] Mean for samples taken 0, 2, and 4 hr. after feeding.

(Table 1) and time after feeding. Ethanol tended to depress the concentration of ammonia nitrogen in the flasks fermented in vitro, as shown in Table 2. Ammonia nitrogen increased with fermentation time in these flasks. Therefore, there was little or no net synthesis of protein, and ethanol apparently depressed ammonia formation. When the incorporation of sulfate sulfur^{35} was utilized as a measure of protein synthesis (Table 3), it was found that methionine,

TABLE 2
Ammonia formation during in vitro fermentation

Substrates	Control	Ethanol
	(mg. % NH_3 N in media)	
Corn	10.4	10.2
Corn + ammonium acetate	22.7	22.2
Hay + ammonium acetate	52.1	51.9
Mean of all substrates	28.4	28.1[a]

[a] P less than 10%.

cystine, and/or cysteine and glutathione synthesis were all slightly depressed by the presence of ethanol. In cystine formation, this was significant at the 5% level.

Ethanol depressed blood urea nitrogen in the trials with the fistulated cows from a mean of 32.9 mg. % for the control to 30.6 mg. %. These cows were emaciated throughout the experiment. A mean blood urea-N content of 8.4 mg. % was observed with the ethanol rations in the balance trial versus 6.8 mg. % with the control rations. Both differences approached significance at the 5% con-

TABLE 3
Microcuries inorganic S^{35}–sulfate incorporated into amino acids

	Methionine[a]	Cysteine[a]	Glutathione[a]
Ethanol	2.4	11.0[b]	6.4
Control	3.4	14.9	7.6

[a] Compounds plus their decomposition products.
[b] Significantly lower than control at 5% level.

fidence level. Bates *et al.* (2) found that 6% ethanol in molasses augmented blood urea when fed ad libitum. The total ethanol ingestion under the conditions of Bates *et al.* (2) was about one-half that in the trials reported here. The inconsistency in blood urea might be due either to the method of administration of the ethanol or to the emaciation noted in the fistulated cows.

The total gas production in vitro was depressed by ethanol in each of the five trials (Table 4). Gas was collected for the 1st hr., then was collected separately for the last 2 hr. of this fermentation. The CO_2 to methane ratio was always much higher for the 1st hr. than for the last part of the fermentation. Ethanol tended to depress methane slightly more than CO_2 production. This tendency occurred only during the last part of the fermentation and was not significant.

Although ethanol disappears from the rumen with a half-time of about 2 hr., when the ethanol concentration was determined during in vitro fermentations before and after incubation, there were no indications of a disappearance of ethanol. Therefore, ethanol was not fermented by the rumen bacteria. This is surprising, since it is known that bacterial species present in the rumen do utilize ethanol for growth (3) and energy (10) under certain circumstances. It would appear that the ethanol did affect the metabolism of the rumen bacteria, particularly when it was in concentrations of 500 mg. %. However, at the level fed, this concentration was present in the rumen only a very short time, if at all.

TABLE 4

Ratio of CO_2 to CH_4 in gas produced during 3.5 hr. fermentation of rumen material in vitro[a]

	Control	Ethanol	Probability[b]
Total gas *(ml.)*	272	225	15%
Ratio CO_2/CH_4	4.6	4.8	>50%

[a] 250 mg. % to 500 mg. % ethanol in media.
[b] Five trials, probability of a larger F to the nearest 5%.

Where effects on metabolism were noted, particularly in the gas production, the effect of the ethanol was to decrease fermentation rates. Bates (2) observed a slight depression of the rumen volatile fatty acids with the exception of valeric acid by ethanol feeding; Head (5) observed an augmentation of the rumen acetic acid concentration with ethanol feeding. The results of this experiment (Table 1) confirm those of Bates (2). Ethanol also tended to depress the amount of digesta in the rumen. The mean amounts of wet digesta expressed as percentage of body weight were 16.0 for the control and 14.6 for the ethanol-treated cows. On the dry basis, these values were reduced to 1.9% for the control and 1.8% for the ethanol-treated cows.

Ethanol tended to depress digestibility and nitrogen retention (Table 5). These effects were not significant, although the depression of crude protein digestibility approached significance at the 5% confidence level. These results are in agreement with those of Balch *et al.* (1).

TABLE 5

Results of digestibility and nitrogen balance trials

	Control		Ethanol	
	Low protein	Urea	Low protein	Urea
Digestibility *(%)*				
Organic matter	64.0	67.2	62.2	65.5
Crude protein	51.2	65.9	45.6	64.5
Nitrogen balance				
Daily intake *(g.)*	87.8	124.1	87.8	124.1
Daily retention *(g.)*	25.6	48.6	19.2	50.6

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EXTENT OF DIGESTION IN THE RUMEN-RETICULUM-OMASUM OF GOATS

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SUMMARY

The extent of digestion in the rumen-reticulum-omasum was studied, using goats whose forestomachs were separated from the abomasum, in order to determine quantitatively the amount of omasal ingesta. Eleven digestion trials were conducted using three such forestomach-separated goats giving various kinds of feeds, e.g. roughage alone, hay with various concentrates, and a nonprotein diet.

The absorption of digestible organic matter from the forestomach ranged from 61.7 to 85.4%. The apparent digestibility coefficients of crude protein in the forestomach varied widely and were positively related to dietary protein levels. The absorption of digestible protein in the forestomach ranged from −20.0 to 52.1%. In animals fed roughage alone, 61.9 to 73.2% of digestible ether-extract disappeared from the forestomach but, in animals fed concentrates and hay, smaller amounts (−2.9 to 28.8%) were absorbed from the forestomach. Almost all of the digestible crude fiber disappeared from the forestomach and 65.7–96.9% of the digestible NFE was absorbed from the forestomach. Except in one trial, crude ash showed negative values, due to the large amount of saliva secreted into the rumen.

The true digestibility of protein in the forestomach, and the amount of saliva secreted, also were studied.

The importance of the digestion processes taking place in the rumen has long been recognized but, as yet, there are few quantitative data available. In previous work, samples of rumen contents for subsequent analysis have been obtained by complete removal of the rumen contents from fistulated animals at various time intervals (7, 8), from samples of the reticulo-rumen digesta taken intermittently near the omasal orifice of fistulated animals (1), or by slaughter of the animal at a predetermined time after feeding and subsequent sampling of the gut contents (5). The above procedures included the use either of external or of internal digestibility indicators, chromic oxide or lignin. Because of the varying rates of passage of these indicators through the alimentary tract (10, 11, 12), the data are subject to uncertain errors. Therefore, it would seem more desirable, in estimating the extent of digestion in the rumen, to quantitatively collect alimentary tract contents upon entry into the omasum or abomasum, by establishment of an appropriate fistula. Such a fistula appeared feasible in light of the recent report by Conver *et al.* (2), but their duodenal fistula would not be appropriate for studies dealing with rumen digestibility.

The present work was concerned with quantitative estimation of rumen digestibility in goats by collecting all alimentary tract contents upon entry into the abomsum via a fistula establishment between the abomasum and omasum.

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EXPERIMENTAL PROCEDURE

Animal. Three female goats of a native Japanese breed were used. This is a light-weight, early-maturing breed and the animals are easy to manage after an operation. Their ages were 3 yr. (Goat A) and 18 mo. (Goats B and C) and their body weights were 30, 22, and 21 kg., respectively, at the start of this experiment. Female goats were used, since they were suitable for the separate collection of feces, urine, and the omasal ingesta, as the new outlet of the omasum was located on the ventral surface of the body.

Surgery. After two days of fasting, a goat was anesthetized by intravenous injection of 10 ml. of 0.4% of Rabonal.[1] An incision about 15 to 20 cm. was made along the mid-line, and the juncture of the omasum and abomasum was withdrawn from the body cavity. The omasum was cut from the abomasum, avoiding the main blood vessels and nerves. A new outlet for the omasum about 1 cm. in diameter was located and sutured along the mid-line. After completely suturing the open end of the abomasum, it was located and sutured to the right abdominal wall about 15 cm. from the mid-line. A second-stage operation was performed two days later, to make a new inlet for the abomasum. A polyvinyl chloride plastic pipe, 1 cm. in diameter, was inserted into the abomasum through which omasal ingesta and other nutrients were to be introduced (Figure 1). The length of the pipe to stay inside daily varied from 4 to 8 cm., to avoid the continuous stimulation on the same spot of the abomasum wall. After opening the

[1] Rabonal contains 0.5 g. of thiopental sodium per gram.

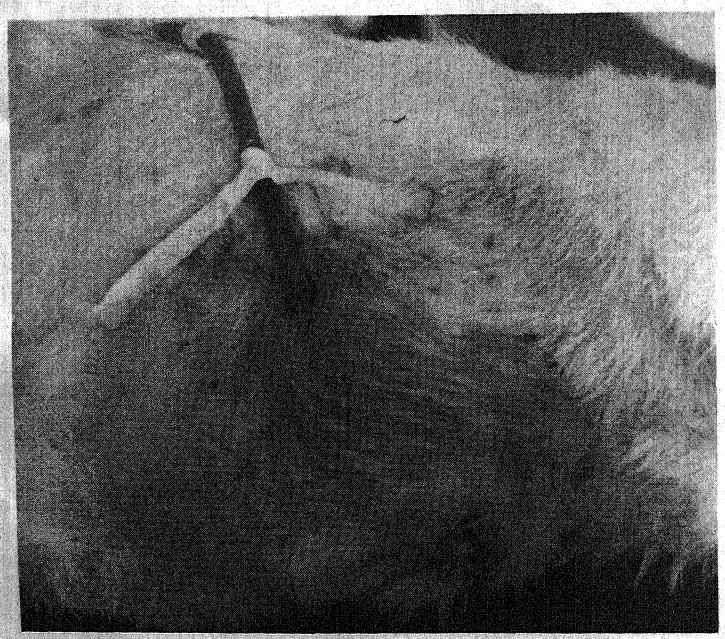


FIG. 1. Omasal outlet and abomasal inlet with vinyl pipe.

new inlet, the feed and water were given gradually. In this report, the goats on which this operation was performed were called the forestomach-separated goats. Further details of the operation will be reported in a separate paper.

Management of the forestomach-separated goats. The goats recovered health and appetite after about seven days, when they were equipped with the apparatus for collecting the omasal ingesta (Figure 2) and placed in metabolism cages. To aid in the total collection of omasal ingesta, the movement of the goats was restricted to a minimum. It was observed that the omasal content did not flow out from the omasal outlet but was pushed out by the action of the omasum.

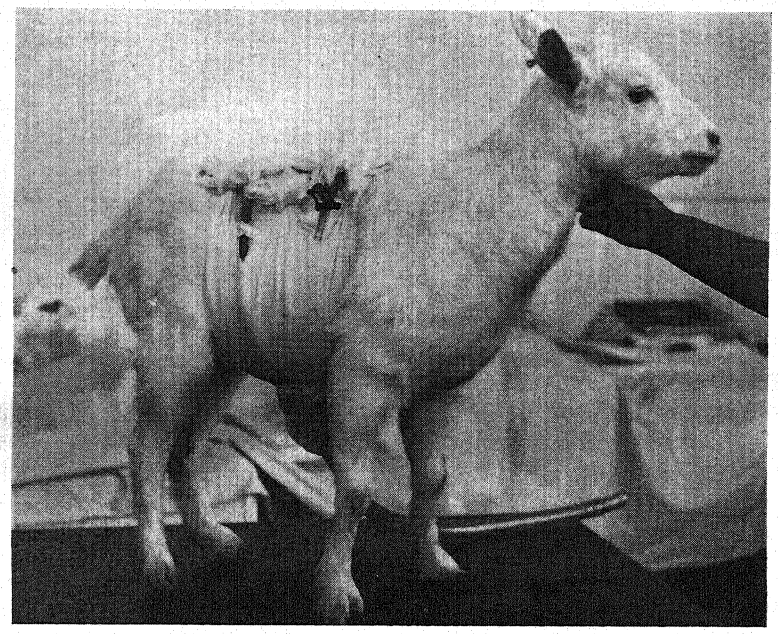


FIG. 2. The forestomach-separated goat with apparatus for collection of the omasal ingesta.

So the words omasal ingesta in this report do not mean the omasal content but the material which has completely gone through the digestive process in the forestomach. Omasal ingesta was poured into the beaker placed under the metabolism cage. The amount was weighed hourly, warmed at 40° C., and poured into the abomasum through the vinyl pipe. Intervals longer than an hour caused the feces to become soft and a tendency for diarrhea to develop. The apparatus for collecting omasal ingesta was renewed once a day.

Digestion trials. Digestion trials were conducted as in the conventional method, except in the treatment of the omasal ingesta. The management of the forestomach-separated goats was so tedious that the digestion trials were conducted continuously, in the order shown in Table 1. The periods of prefeeding and feces collection were shortened to seven days, except in Trial 11, when the prefeeding period was six days and feces collection period was four days. During

TABLE 1

Design and duration of experiment, weights of feed, and omasal ingesta and feces produced during the collection period

Trial No.	Goat	Pre-feeding	Collection	Feed consumed[a]		Omasal ingesta Fresh	Omasal ingesta Dry[a]	Feces[a, b]
		(days)	*(days)*			*(g.)*		
1	A	7	7	Conc.	1,538	33,684	1,908	1,345
				Hay	1,834			
2	A	7	7	Rye (green fodder)	4,002	41,713	1,939	1,135
3	B	7	7	Conc.	1,425	26,244	2,203	1,332
				Hay	1,757			
4	C	7	7	Conc.	1,741	28,678	2,204	1,353
				Hay	1,757			
5	B	7	7	Hay	2,542	26,491	1,580	1,273
6	C	7	7	Hay	2,839	30,514	1,878	1,422
7	B	7	6	Conc.	639	18,204	1,182	693
				Hay	1,294			
8	C	7	6	Conc.	2,234	26,575	1,710	940
				Hay	965			
9	B	7	7	Conc.	960	27,420	1,951	1,340
				Hay	1,974			
10	C	7	7	Conc.	1,089	30,092	2,061	1,553
				Hay	2,238			
11	B	6	4	Conc.	761	9,136	358	138

[a] Dry matter.
[b] These values were corrected with calculation.

the digestion trials, the sampling was performed hourly 24 times a day; the omasal ingesta was collected in the beaker, which was mixed thoroughly every time before one-tenth of it was removed. These samples were dried immediately in a circulating air dryer at 55° C., then weighed for chemical analysis. It was the aim of the authors that these samples would be the same as those obtained by collecting the entire omasal ingesta throughout the test period, homogenizing, and taking one-tenth of it for a sample. The amounts of feces shown in Table 1 were adjusted by a factor of ten-ninths, so as to equal the amounts of feces obtained when the omasal ingesta was not removed.

In Trials 2, 5, and 6, the goats were fed on roughage only, and in Trial 11 they were fed a semisynthetic nonprotein ration. In all other trials, a low-quality hay of predominantly orchard grass and red clover was fed with various concentrate mixtures. The composition of the concentrate mixtures is given in Table 2. They were altered to obtain various fiber-protein-urea-carbohydrate ratios.

As the goats in Trials 5, 6, and 11 were given relatively little or no protein, 2 g. of casein was administered into the abomasum with the remainder of omasal ingesta hourly, i.e., 48 g. per day. In these trials, the digestibility coefficients of protein were calculated on the assumption that this casein was absorbed completely from the gut. Water was given ad libitum.

The entire feces was collected before the morning feeding, and the residues of feed and water were weighed and measured. A ration containing no nitrogen was given to a goat in Trial 11, which was performed to determine the true digestibility of the protein in the forestomach. The sources of endogenous nitro-

TABLE 2

Composition of concentrate mixtures fed during each specified trial

Ingredient	Trial No.					
	1	3 and 4	7 and 8	9	10	11
	(%)					
Wheat bran	35	35	—	47	47	—
Rice bran	35	30	—	—	47	—
Ground corn	17	10	—	47	—	—
Soybean meal[a]	10	10	—	—	—	—
$CaCO_3$	2	2	—	2.5	2.5	—
NaCl	1	1	—	1	1	—
Dried skimmilk	—	12	—	—	—	—
Casein	—	—	23	—	—	—
Sweet potato powder	—	—	30	—	—	—
Corn starch	—	—	30	—	—	40
Sucrose	—	—	7	—	—	28
Soybean oil	—	—	5	—	—	6
Mineral mixture[b]	—	—	5	—	—	5.9
Vitamin mixture[c]	—	—	—	—	—	0.1
Urea	—	—	—	2.5	2.5	—
Filter paper	—	—	—	—	—	20

[a] Solvent extracted.

[b] Mineral mixture $CaHPO_4 \cdot 2H_2O$ 11.0%; $CaCO_3$ 16.0%; K_2HPO_4 27.0%; $MgSO_4 \cdot 7H_2O$ 15.0%; Na_2SO_4 22.6%; $FeC_6H_5O_7$ 2.5%; $MnSO_4 \cdot 4H_2O$ 0.7%; KI 0.08%; $ZnCl_2$ 0.02%; $CuSO_4 \cdot 5H_2O$ 0.03%; $COSO_4 \cdot 7H_2O$ 0.07%.

[c] Each 1 g. contains vitamin A, 2,500 I.U., vitamin D_2, 200 I.U., vitamin E, 1.0 mg., vitamin B_{12}, 1 γ, and so on.

gen may be saliva, along with rumen-wall cellular material, realimentation of liquids, etc. The authors intended to attempt the estimation of saliva, definitely the major source of endogenous nitrogen, not counting the probable water secretion and absorption taking place there. The method of estimation was as follows: (fresh weight of ingesta passing through the omasum) − (weight of water taken plus dry weight of omasal ingesta).

Chemical analysis. Moisture, crude protein, ether-extract, crude fiber, NFE, and crude ash were determined by the A.O.A.C. methods.

RESULTS

The total amount of feed consumed, omasal ingesta and feces produced during the feces collection periods are shown in Table 1. The amount of feed consumed by each goat was variable between trials, due to the palatability of feed and the condition of the goats, though intake during each trial was nearly constant.

The results of chemical analyses of each feed, omasal ingesta, and feces are shown in Table 3. The contents of protein, ether-extract, and crude ash in omasal ingesta were always higher than the corresponding values of both feed and feces. The opposite tendency was shown for the content of organic matter, crude fiber, and NFE. These tendencies seem to mean that the former are chiefly absorbed from the hind gut, and the latter from the forestomach.

In these observations, the apparent digestibility coefficients of crude protein in the forestomachs varied widely from −8.2 to 38.5%, as shown in Table 4. The digestibility coefficients of ether-extract in the forestomachs were low or negative,

TABLE 3

Chemical compositions of feed, omasal ingesta, and feces in each digestion trial

Trial No.		Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
		(%)					
	Feed	86.61	14.51	2.30	18.58	51.22	13.39
1	Omasal ingesta	74.85	24.16	4.01	12.90	33.78	25.15
	Feces	78.38	18.23	2.71	17.52	39.92	21.62
	Feed	90.14	24.78	5.09	21.81	38.46	9.86
2	Omasal ingesta	74.40	31.47	5.44	9.93	27.56	25.60
	Feces	84.77	22.80	6.14	15.58	40.25	15.23
	Feed	87.34	16.94	2.51	17.30	50.59	12.66
3	Omasal ingesta	78.29	22.10	3.70	12.31	40.18	21.71
	Feces	79.88	18.72	2.56	20.72	37.88	20.12
	Feed	87.71	17.64	2.53	16.30	51.24	12.29
4	Omasal ingesta	75.12	25.02	3.99	13.03	33.08	24.88
	Feces	79.30	20.01	2.42	20.64	36.24	20.70
	Feed	85.81	11.94	2.30	25.33	46.24	14.19
5	Omasal ingesta	76.96	19.26	2.57	18.12	37.01	23.04
	Feces	82.52	13.43	2.31	22.32	44.46	17.48
	Feed	85.81	11.94	2.30	25.33	46.24	14.19
6	Omasal ingesta	79.30	19.53	2.42	18.38	38.97	20.70
	Feces	82.71	14.09	2.51	22.29	43.82	17.29
	Feed	87.79	16.12	3.81	17.43	50.43	12.21
7	Omasal ingesta	77.10	23.17	5.72	12.24	35.97	22.90
	Feces	80.22	19.81	2.45	16.49	41.47	19.78
	Feed	89.98	20.71	5.46	8.74	55.07	10.02
8	Omasal ingesta	77.78	29.26	7.81	5.75	34.96	22.22
	Feces	81.70	26.87	3.20	10.90	40.73	18.30
	Feed	87.94	14.90	2.87	19.59	50.58	12.06
9	Omasal ingesta	77.23	22.61	3.71	14.52	36.39	22.77
	Feces	81.49	17.55	2.89	20.31	40.74	18.51
	Feed	85.92	16.31	2.55	20.89	46.17	14.08
10	Omasal ingesta	74.06	20.38	3.71	15.37	34.60	25.94
	Feces	79.80	15.66	2.57	21.47	40.10	20.20
	Feed	95.16	1.56	6.26	20.18	67.16	4.84
11	Omasal ingesta	76.09	15.67	7.56	20.69	32.17	23.91
	Feces	90.78	17.24	2.50	40.03	31.01	9.22

except when roughage was fed alone. For crude fiber, almost the same values were obtained in the forestomachs as in the entire gut. NFE was digested to a markedly greater degree in the forestomach than in the hind gut. Except in Trial 6, the digestibility coefficients of crude ash in the forestomachs were always negative, due to the large amount of salivary ash secreted into the rumen.

To understand clearly the extent of digestion in the forestomachs, the ratio of the digestibility coefficient of each component in the forestomach to that in the entire digestive tract is shown in Table 5 (61.7 to 85.4% of digestible organic matter disappeared in the forestomachs). The disappearance of digestible protein in the forestomachs ranged from −20.0 to 52.1%, except in the nonprotein feeding trial. When goats were fed either on roughage alone or on a nonprotein diet, 46.5 to 73.2% of digestible ether-extract disappeared from the forestomachs. In contrast, when they were fed on hay with various amounts of concentrate, lesser amounts were absorbed from the forestomachs, ranging from −2.9 to 28.8%. The disappearance of fiber in the forestomachs ranged from 93.0 to 104.6%.

To estimate the true digestibility of protein in the forestomach, the amount of saliva secreted was determined, and the results are shown in Table 6. The

TABLE 4

Digestibility coefficients of feed, in the forestomach and in the entire gut

Trial No.		Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
		(%)					
1	Forestomach	51.1	5.8	1.4	60.7	62.7	—6.3
	Entire gut	63.9	49.9	53.0	62.4	77.2	35.6
2	Forestomach	60.0	38.5	48.2	78.0	65.3	—25.9
	Entire gut	73.4	73.9	65.8	79.8	70.3	56.2
3	Forestomach	38.1	10.0	—1.6	50.9	45.2	—18.2
	Entire gut	61.9	53.9	57.5	50.1	68.7	33.8
4	Forestomach	46.1	10.7	1.5	49.6	59.2	—27.6
	Entire gut	65.1	56.1	62.9	51.0	72.8	35.9
5	Forestomach	44.3	—0.2	30.8	55.5	50.2	—0.8
	Entire gut	51.9	43.7	49.8	55.9	51.9	38.4
6	Forestomach	38.9	—8.2	30.5	52.0	44.3	3.5
	Entire gut	51.7	40.9	45.9	55.9	52.5	26.4
7	Forestomach	43.6	7.7	3.6	54.9	54.3	—20.4
	Entire gut	66.0	59.0	79.8	54.8	70.3	33.5
8	Forestomach	53.8	24.7	23.9	64.5	66.1	—18.7
	Entire gut	73.3	62.0	82.9	63.1	78.3	46.3
9	Forestomach	41.6	—1.0	13.8	50.7	52.2	—25.6
	Entire gut	57.7	46.2	53.9	52.6	63.2	29.9
10	Forestomach	46.6	22.6	9.9	54.4	53.6	—14.1
	Entire gut	56.7	55.2	52.9	52.0	59.5	33.1
11	Foretomach	62.4	—371.5	43.1	51.8	77.5	—132.1
	Entire gut	82.6	—100.5	92.7	63.9	91.6	65.4

TABLE 5

Ratio of disappearance of each digestible component in the forestomach to that of the entire gut

Trial No.	Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
	(%)					
1	80.0	11.7	2.6	97.4	81.2	—19.0
2	81.8	52.1	73.2	97.7	92.9	—46.0
3	61.7	18.5	—2.9	101.6	65.7	—53.9
4	70.8	19.0	2.4	97.3	81.4	—76.8
5	85.4	—0.4	61.9	99.4	96.9	—2.2
6	75.2	—20.0	66.3	93.0	84.3	13.4
7	66.2	13.1	4.6	100.2	77.2	—61.0
8	73.4	39.8	28.8	102.4	84.4	—40.4
9	72.1	—2.3	25.6	96.3	82.5	—85.7
10	82.3	40.9	18.8	104.6	90.1	—42.7
11	75.5	—369.6	46.5	81.0	84.6	—202.1

amount of saliva secreted paralleled the intake of hay. The results of Trial 11 permit the calculation of the nitrogen content of saliva (13.65 mg. of nitrogen was contained in 100 ml. of saliva). Based on this value, the true digestibility of protein in the forestomach was calculated, with the results shown in Table 6. It is found that 52.6–87.0% of digestible protein of feed was absorbed from the forestomach.

DISCUSSION

Initially, the authors were concerned that some difference might result in the digestibility values obtained from the forestomach-separated goats and those from untreated goats. Such apprehensions occurred for the following reasons:

TABLE 6

Amount of saliva secreted and the true digestibility of protein in the forestomach in each trial

Trial No.	Water taken[a]		Saliva[a] secreted	True digestibility of protein in the forestomach	True digestibility of protein in the forestomach/digestible protein in feed
	Water drunk	Water contained in feed			
	(ml.)	*(g.)*	*(g.)*	*(%)*	*(%)*
1	938	78	3,521	32.9	66.0
2	.....	3,586	2,094	46.5	62.9
3	423	53	2,957	30.6	56.8
4	747	59	2,974	29.5	52.6
5	86	41	3,430	42.0	87.0
6	321	46	3,722	32.7	79.9
7	305	38	2,494	37.8	64.1
8	640	64	3,473	41.3	66.6
9	226	52	3,359	27.7	60.0
10	283	58	3,662	46.5	84.2
11	106	16	2,073	.....	.....

[a] Average per day.

(a) Since the digestive tract was incised between the omasum and abomasum, the content of the gut was inevitably exposed to the air midway in digestion. (b) In untreated goats, the digesta moves through the digestive tract continuously, but in the operated goats comparatively large amounts of digesta were poured into the abomasum hourly. (c) Moreover, the activity of the experimental animal was limited rigidly. A supplementary experiment (9) was conducted to determine whether there were any differences in digestibility between the untreated and the operated goats. As a control for Trial 2, an untreated goat was fed the same green rye as in Trial 2. The results were: digestibility coefficient of organic matter 23.8%, crude protein 77.1%, ether extract 63.4%, crude fiber 74.9%, NFE 72.5%, and crude ash 56.4%. As a control for Trial 4, the same feed was given to an untreated goat. The results were: digestibility coefficient of organic matter 64.4%, crude protein 62.5%, ether-extract 61.6%, crude fiber 51.6%, NFE 69.3%, and crude ash 29.7%. These results show there was little difference between the figures obtained with the operated animal and the untreated one (*cf.* Table 4). Accordingly, it must be concluded that the extent of digestion in the operated goats was essentially unchanged from that of untreated goats.

Concerning the true digestibility of protein, the range of absorption of dietary protein from the forestomach was about 52 to 87%. Since ammonia and urea are the principal nitrogen compounds absorbed from the rumen (24), these results indicate that relatively large amounts of protein nitrogen were absorbed from the rumen as ammonia, and that a large proportion of protein was probably converted into microbial protein (13, 14). On the other hand, the apparent digestibility coefficients of protein in the forestomachs were proportional to the protein levels in the feed. In other words, at the lower protein levels it became negative, and at the higher levels it became positive. These facts seem to indicate that at the higher protein levels the nitrogen compounds in feed were partly

absorbed from the rumen as well as the nitrogen secreted in saliva; whereas, at low protein levels no feed protein was absorbed and salivary nitrogen was synthesized into protein. However, the nitrogen of both feed and saliva must be considered as one unit in the rumen.

As previously shown by McNaught and others (16, 17), the conversion rate of ammonia into microbial protein was affected by the presence of carbohydrate and by its quality and quantity. Since the protein level varied in each of these trials, it is impossible to assess the effect of carbohydrate on protein synthesis in the rumen. However, in rations containing small amounts of readily available carbohydrate (Trials 5, 6, and 10), the true digestibility of protein in the forestomachs was about 80% of the digestive protein (Table 6). A large amount of ether-extractable material was absorbed from the forestomach when only roughage was fed; whereas, a small, or negative, amount of it was absorbed when hay and concentrates were fed. This can be explained by the fact that ether-extractable material was formed from carbohydrate and protein by rumen bacteria, as indicated by the work of Hale *et al.* (6, 8). It was reported that the nature of the dietary fat was different from that of the fat in the rumen (20, 21). This indicates that a large portion of the ether-extractable materials are digested and absorbed from the rumen, and that new forms are synthesized.

In these trials, the digestible crude fiber almost completely disappeared while in the rumen, but in previous experiments this was not so and, in certain instances, the largest part of it was digested in the hind gut (1, 6, 8). Perhaps such conclusions were erroneous because the ratio technique was employed.

The disappearance of digestible NFE from the forestomach ranged from 65.7 to 96.9%. NFE is chiefly changed into volatile fatty acids and absorbed from the rumen, but the remaining 3 to 34% of the digestible NFE was, perhaps, absorbed from the small intestine as glucose. A study of the difference between volatile fatty acids and glucose as energy sources for ruminants is an interesting problem.

The amount of saliva secreted and its ash content were so large that the digestibility of crude ash in the forestomach showed negative values except in Trial 6. Since potassium, chloride, sodium, phosphate, etc. are absorbed from the rumen (4, 18, 22, 23), some of the minerals in the feed must have been partly absorbed from the rumen.

Assuming that there was no water secreted into and absorbed from the forestomach, the amount of saliva secreted was estimated as indicated previously and the results are shown in Table 6. Raynard *et al.* (19) reported that water was absorbed from the omasum and Tsuda (24) suggested the possibility of absorption of water from the rumen. The value given in Table 6 for the volume of saliva is actually an estimate of the increased amount of water that occurs during digestion in the forestomach. Since the value obtained in Table 6 approximates the values estimated for salivary volume of sheep (3, 15), this would indicate that there was only a small amount of water exchange occurring in the forestomach.

At the determination of endogenous nitrogen, neither the water exchange in the forestomach nor the variation of the nitrogen contents of saliva was considered. Therefore, the values of true digestibility of protein in the forestomach were only estimated ones. These results of estimations are presented here, in the hope of contributing to the process of clarification of the problem of rumen nitrogen digestion.

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EFFECT OF RECURRING PREGNANCY ON MAMMARY GLAND GROWTH IN MICE [1]

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SUMMARY

It was shown by the increase of total desoxyribosenucleic acid (DNA) that mammary gland growth continues during the latter half of pregnancy. This observation was rather unexpected, since morphological study of lobule-alveolar growth can not distinguish between additional cell growth and the initiation of milk secretion. These data indicate for the first time that cell multiplication continues throughout pregnancy.

The effect of recurring pregnancy on mammary gland growth was studied, employing albino mice bred successively without nursing periods. Maximum growth of the mammary glands was attained in triparous animals. A slight decline in total growth was observed in quadriparous animals. Their age at this time was estimated to be equivalent to the age of cows which begin to decline in yearly milk and fat yields. It was also confirmed that increased mammary gland growth in multiparous animals was more closely related to hormonal stimuli associated with recurring pregnancy and lactation than to increasing body weight. Primiparous mice after 3 mo. of involution had mammary glands containing DNA equivalent to mice pregnant for six days. No variation in average litter size was observed during four consecutive pregnancies.

Milk and fat secretion in dairy cattle has been shown to increase with advancing age up to about 8 yr. The age-production relation has been used extensively to formulate age-correction factors for converting milk and fat production records to their mature equivalent. Two obvious factors are involved in the increase in milk secretion: (a) the increase in body weight up to 8 yr. (7, 9) and (b) recurring pregnancy and lactation. In the analysis of these data it was suggested that recurring pregnancy (and factors associated with pregnancy and lactation) influenced the increase in lactation with age far more than did the increase in body weight. Confirmation of this relation has been provided by an analysis of milk records of Swedish dairy cattle (5). It was shown that milk yield per lactation depends not only on age up to maturity but also on the number of lactations. To reach maximum yield the cows must, on the average, have had at least three pregnancies.

The most obvious factor in recurring pregnancy is growth of the mammary gland. While it has been inferred during the last 30 yr. that additional growth of the mammary gland was stimulated with each pregnancy up to the time of maximum milk yield, no quantitative method of determination of mammary gland growth has been available to prove that additional growth occurred.

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During the past few years, a quantitative method of determining the growth of the mammary gland has been developed (2, 6, 13). It has been shown that the desoxyribosenucleic acid (DNA) content of each mammary gland cell is constant during pregnancy (3). Thus, as the cells multiply, the amount of DNA in the glands increases. A chemical method of increased sensitivity for determination of DNA recently has been described (11). With this method available, it seemed desirable to determine *(1)* the extent of gland growth (as measured by total DNA) during stages of the first pregnancy, and *(2)* whether gland growth was extended by two, three, or four successive pregnancies.

EXPERIMENTAL PROCEDURE

Albino mice fed a standard laboratory feed were bred when weighing about 28–30 g. Pregnancy was dated from the morning a vaginal plug was observed. The multiparous mice were remated immediately after parturition, following the removal of the young. Not all mice conceived immediately, so there was a variable period before the second and successive pregnancies were initiated. At the time indicated in Table 1, the animals were sacrificed, skinned, and three-fourths of the mammary glands of each mouse taken for DNA determination. The remaining one-fourth of the glands was prepared for whole-mount examination, to observe the type of gland development.

The gland tissue for DNA determination was frozen, the fat extracted with hot alcohol and ether and ground to a fine powder. Then, 30 mg. of the gland was extracted twice with 5 ml. of hot 5% trichloroacetic acid to remove the DNA, which was then determined by the Webb-Levy method (11).

Experimental observations. As controls, a group of unmated mice with extensive duct development were used. The average total DNA of the group was 1,704 μg. (Table 1). A certain number of mice, when sacrificed at six and 12 days after coitus, were found not to be pregnant. As coitus stimulates the maintenance of the corpora lutea, i.e., pseudopregnancy (8), the mammary glands of these animals were included in the study. It will be noted that gland growth occurred (DNA, 2,269 μg. per animal) at six days, but was not further increased at 12 days. Since this value is based upon a limited number of animals, the DNA may be increased as a more representative sample is obtained. In the first pregnancy, progressive growth of the mammary gland was observed at six, 12, and 18 days, as indicated by increasing DNA content. The marked increase in DNA from the 12th to the 18th day was unexpected since, on the basis of the morphological observations, it had been assumed that the major part of lobule-alveolar growth occurred during the first half of pregnancy. It was observed, also, that growth of the glands of pregnant animals was greater than that of pseudopregnant animals at the same stages. While further study will be required to confirm these observations, they suggest the possibility that the pregnant state (fetal membranes?) contribute hormones which cause greater mammary gland growth than the ovarian hormones (corpora lutea of pseudopregnancy) alone.

Considering the extent of mammary gland growth produced at the end of the first pregnancy (mean total DNA) as a base, it will be noted during the second pregnancy that there was 28.70% additional growth of mammary gland tissue

TABLE 1

Effect of recurring pregnancy on the mammary gland growth in mice

Reproductive state	Stage of gestation	No. of mice	D.F.F.T.[a] Wt. (mg.) Mean ± S.E.		D.F.F.T. Wt. / Wet wt. of glands (%) Mean ± S.E.		DNA (μg.) / mg. D.F.F.T. Mean ± S.E.		Total DNA (μg.) Mean ± S.E.		Initial body wt. (g.) Mean ± S.E.		Total DNA (μg.) / Initial body wt. (g.) Mean ± S.E.	
Virgin		7	44.0	2.5	4.0	0.7	38.8	3.5	1,704	180	30.8[b]	0.7	54.4[c]	5.1
Nullipara	6 Days after sterile coitus	15	52.5	2.1	5.7	0.4	42.9	0.5	2,269	126	31.6	0.8	71.5	3.8
	12 Days after sterile coitus	5	54.8	3.1	4.3	0.8	39.8	1.4	2,202	159	32.8	1.1	77.8	8.2
Primipara	6 Days of pregnancy	10	63.2	2.4	6.9	0.5	46.3	2.0	2,853	153	31.4	1.0	90.4	4.9
	12 Days of pregnancy	10	80.1	4.6	6.3	0.3	51.4	2.8	4,118	319	32.8	1.0	125.3	9.1
	18 Days of pregnancy	10	121.8	8.6	8.4	0.4	49.4	1.6	5,965	353	32.0	1.3	185.8	7.0
Bipara	18 Days of pregnancy	10	154.0	5.9	9.8	0.5	49.3	1.9	7,677	438	33.4	1.2	235.6	20.2
Tripara	18 Days of pregnancy	10	165.9	10.9	9.3	0.3	45.4	0.8	7,730	497	36.1	1.4	215.0	9.2
Quadripara	18 Days of pregnancy	10	146.0	8.6	9.2	0.2	46.5	1.2	6,982	366	36.9	0.9	188.5	6.3
Primipara	Sterile over 3 mo. after first parturition	7	69.3	8.0	6.5	0.9	40.2	3.9	2,720	286	32.2[b]	1.7	83.8[c]	7.9

[a] D.F.F.T. = Dry, fat-free tissue.
[b] Final body weight.
[c] Total DNA/final body weight.

(total DNA) and during the third pregnancy an additional further slight increase. Thus, the total additional mammary gland growth induced by two successive periods of pregnancy amounted to an increase of 29.59% over that induced by the first pregnancy. To the extent that additional epithelial cells present represent additional capacity to secrete milk by the tripara mice, it is assumed that their lactational capacity would thus be increased by about 30% as a result of two additional pregnancies. Since the average body weight of the mice at the beginning of the first pregnancy was 32.0 g. and of the tripara mice 36.1 g., the additional mammary gland growth induced was only slightly related to the increasing body weight. In other words, there is a marked increase in total DNA per gram increase in body weight in the bipara mice. It will be noted, also, that the weight ratio of dry fat-free tissue to wet tissue of the multiparous mice was greater than that of the primiparous mice. With only slightly higher nutritional maintenance requirement of the bipara and tripara animals, the increased lactational capacity of the animals with larger mammary glands would increase the nutritional efficiency for milk production of the latter groups.

The group of quadripara mice showed a 9.68% decline in average total DNA, in comparison with the tripara group. The decline in capacity for mammary gland growth is believed to represent a decline in capacity for the secretion of the hormones essential (directly and indirectly) for mammary gland growth. This compares with the gradual decline in yearly milk and fat production of dairy cattle after peak production is reached at 7 or 8 yr. Brody (1) reported that 1 mo. in the mouse was equivalent to 15.3 mo. in the cow. The quadripara mice in this experiment were in range of 5.5–6.5 mo. of age, averaging 6 mo. at the end of their pregnancies. This is equivalent to cows 85 to 100 mo. of age (see Brody's Figure 19.7). Thus, the quadripara mice were of an age comparable to cattle which begin to decline in yearly milk yield. It has been known that thyroid function has a relation to the age, and that features of the hypothyroid state are similar to some of the features of senescence. Recently, it has been reported that the thyroxine secretion rate of rats decreases with advancing age (12). Unpublished preliminary data also showed that thyroxine secretion rate per unit of body weight in aged mice is lower than in younger animals by radioactive replacement technique (10). Therefore, the decline of mammary gland growth in quadripara mice may be due to a decline of thyroxine secretion rate as well as pituitary activity and other physiological functions. In primipara mice which were not bred again for 3 mo. or more, there was observed involution of the mammary glands to an extent comparable to that observed at the sixth day of pregnancy. Even in animals at 25 days after parturition, without recurring pregnancy and nursing, there was marked involution of mammary alveolar tissue (not included in Table 1).

Ingram *et al.* (4) has recently shown that age of mice does not influence litter size up to the fourth; however, a decline in litter size occurs thereafter. The results of the present experiment show no direct relationship between the litter size and mammary gland growth, since average litter size in all groups was constant, ranging from 9.0 to 9.5.

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EFFECTS OF VARIOUS AMOUNTS OF CHLORTETRACYCLINE IN THE RATIONS OF LACTATING DAIRY CATTLE

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SUMMARY

Aureomycin[1] (chlortetracycline) was fed to lactating dairy cattle at levels of 0, 0.1, 0.5, and 1.0 mg. per pound of body weight per cow per day for a period of 2 wk. Although two of the four cows in each of the two groups receiving the higher doses showed slightly depressed appetites shortly after the trial was started, feed consumption returned to normal after a brief period of adjustment. During this adjustment period, milk production also was reduced, but generally returned to similar relative levels of the controls within a few days. No marked clinical effects were noted in the group receiving 0.1 mg. per pound of body weight, and milk from these cows was negative for the presence of the antibiotic. Slight amounts of chlortetracycline were found in the milk of cows on higher feeding levels and these slight amounts were eliminated from all cows' milk by 48 hr. after the feeding of the antibiotic had been discontinued.

Early work by Bell *et al.* (2) in beef cattle and Colby *et al.* (3) in sheep appeared to support the theory that oral administration of antibiotics to ruminating cattle or sheep would be harmful, since bacteria are essential to the digestive mechanism of these animals. Later workers (1, 5, 8, and 15) reported that Aureomycin (chlortetracycline) could be fed without harmful effect to ruminating cattle at levels in excess of the total dose commonly fed to calves. These later reports renewed interest in the application of antibiotics for the feeding of ruminating animals, and much work with beef cattle and sheep has been reported in the literature in the last few years. The acceptance of antibiotics in fattening rations for beef cattle and sheep is now widespread and is a common, although not universal, practice in commercial operations.

Loosli and Warner (11), Rusoff and Haq (14), and Jacobson *et al.* (9) reported that levels of 130–700 mg. of chlortetracycline per cow per day had no harmful effect on feed consumption or milk production. However, relatively little interest was shown in adapting the results of beef cattle work to dairy cattle, since the major result—improved weight gains—is not of primary importance in commercial dairy herds. Also, it was not known what effect the feeding of antibiotics would have on milk intended for cheese manufacturing, although Loosli and Warner (11) and Martin *et al.* (12) reported that feeding relatively high levels of chlortetracycline to dairy cattle did not interfere with the use of milk for the manufacture of cheese.

Public health officials were concerned about the possible presence of antibiotics in the milk and the effect they might have on persons consuming the milk. Henderson *et al.* (7) studied this question and their work indicated that the feeding of 0.1 mg. of chlortetracycline per pound of body weight per cow per day did not produce measurable amounts of antibiotic either in milk or in the blood. This

[1] The trademark of the American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

is the dose which has been permitted for commercial use by the Food and Drug Administration. The object of our investigation was to repeat the work of Henderson *et al.* (7), who fed several levels of chlortetracycline, to provide additional information on this subject for federal and state authorities who wished to see the work repeated. The major emphasis in this report will be placed on the levels of antibiotic found in the milk from cows fed three different levels of chlortetracycline.

EXPERIMENTAL PROCEDURE

Sixteen normal lactating dairy cows were allotted to one of four treatment groups, with four cows being allotted to each group. Aureomycin (chlortetracycline) Crumbles, containing 2.0 g. of the antibiotic per pound, was added to the rations of the cows in each group, at levels to provide the following daily doses of antibiotic:

Group 1—Control. No chlortetracycline.

Group 2—0.1 mg. chlortetracycline per pound of body weight per cow per day.

Group 3—0.5 mg. chlortetracycline per pound of body weight per cow per day.

Group 4—1.0 mg. chlortetracycline per pound of body weight per cow per day.

The antibiotic was placed in the concentrate portion of the ration, with one-half the dose fed at each of the two daily feedings. The antibiotic feeding was conducted for a period of 2 wk.

Observations on total feed consumption were made during this period. Total pounds of concentrate and hay consumed each day and the speed at which they were consumed were recorded. Total consumption of feed revealed the effect, if any, on appetite; whereas, this factor plus speed of consumption, especially of the concentrate, indicated whether palatability of the ration was affected.

The effect on milk production and any evidence of toxicity or digestive disturbances caused by the trial procedure were recorded. Milk samples were obtained from each cow at the start of the experiment. Samples also were taken from each of the treated cows at 24-hr. intervals, continuing for three days after the feeding of the antibiotic was stopped. Antibiotic levels in the milk were determined by a modification of the Food and Drug Administration cylinder-plate technique (4), which has a sensitivity level of 0.01 γ/ml.

RESULTS

On the second day of antibiotic feeding, two cows on each of the 0.5 and 1.0 mg. feeding levels refused part of the grain ration. On the third day, one of these cows in each group was still off-feed to some degree. The feeding procedure was rechecked and it was found that these—as well as the other cows in the same groups—were receiving 1.0 and 2.0 mg. per pound of body weight, respectively. The dose was, therefore, readjusted to that planned in the original design

of the experiment. All animals quickly returned to full feed consumption thereafter; no apparent effect on palatability of the feed was noted.

Cow 777 became ill during the experiment. Since this animal was not eating grain or roughage, its milk production was not included and the milk was negative for the antibiotic. The clinical symptoms were those of acetonemia, and finding acetone in the urine and milk confirmed this diagnosis. This condition did not appear to be a result of the treatment, since no ill effects from feeding antibiotic have been reported by other workers (1, 5, 6, 8, 9, 11, 12, 14, 15) who fed both lower and higher, as well as similar, doses of chlortetracycline.

No signs of toxicity or other untoward effects directly attributable to the antibiotic were observed, other than the temporary reduction in feed intake referred to earlier and the accompanying transitory reduction in milk production.

Figure 1 shows the average daily milk production for each group from four days before antibiotic feeding through four days after it was eliminated from the ration. Milk production in all groups dropped on the third day after chlortetracycline feeding started. The experiment was conducted in the summer and the weather was extremely hot on this day. However, while the drop in the group receiving 0.1 mg. of chlortetracycline per pound of body weight per cow per day was similar to that of the controls, the lowered production in the other groups was of a greater magnitude and was apparently related to the administration of the antibiotic.

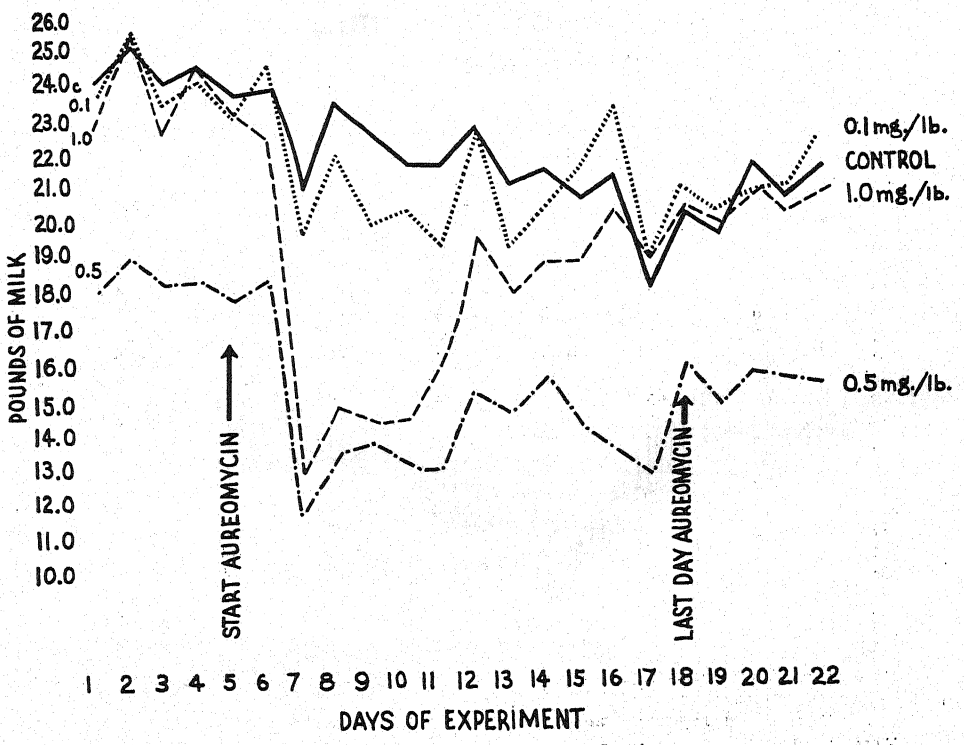


FIG. 1. Average daily milk production.

In the group receiving 1.0 mg. of chlortetracycline per pound of body weight per day, production returned within a few days to a level comparable to that of the control and the low level groups. The cows receiving 0.5 mg. chlortetracycline per pound per day increased in production gradually, but did not come up to the same production as other cattle until some time after the experiment was concluded. Their initial production was below that of the other cows and they returned to producing comparable levels in only a slightly longer time than the others.

The levels of chlortetracycline in the milk are shown in Table 1. Milk from the controls and all of the treated cows was negative at the start of the experiment and is, therefore, not included in the table. Some of the early readings in the 0.5 and 1.0 mg. treatment groups were high because of the higher actual dosage, but later readings are considered more typical and are in agreement with the work of Henderson (6). All milk was negative 24 hr. after the removal of the antibiotic from the diet, with the exception of samples from two cows on each of the high levels of treatment; their milk was negative after 48 hr.

DISCUSSION

The transitory depression in feed intake reported is similar to that occasionally seen for beef cattle shortly after the antibiotic is introduced in the ration. In all probability, this reflects an adjustment of the rumen flora. This was not

TABLE 1

Amounts of chlortetracycline in milk during 14 days of continuous feeding

Sampling time in hours after start of medication	Feed treatment[a]							
	0.5 mg. chlortetracycline/lb body weight/day				1.0 mg. chlortetracycline/lb body weight/day			
	Cow No.				Cow No.			
	772	776	777[b]	778	764	765	766	767
	(μg/ml milk)							
0	0	0	0	0	0	0	0	0
24	0.03	0.02	0.03	0.01	0.04	0.06	0.04	0.05
48	0.10	0.05	0.03	0.04	0.13	0.20	0.11	0.14
72	0.11	0.21	0.03	0.05	0.17	0.23	0.20	0.13
96	0.05	0.05	0.03	0.04	0.14	0.16	0.20	0.05
120	0.03	0.03	0.01	0.02	0.03	0.13	0.17	0.04
144	0.06	0.05	0	0.04	0.08	0.10	0.21	0.04
168	0.07	0.05	0	0.03	0.08	0.11	0.14	0.05
192	0.07	0.06	0	0.03	0.06	0.08	0.07	0.06
216	0.07	0.05	0	0.02	0.07	0.07	0.08	0.05
240	0.04	0.02	0	0.02	0.06	0.04	0.11	0.12
264	0.06	0.06	0	0.02	0.06	0.09	0.13	0.06
288	0.09	0.03	0	0.02	0.07	0.05	0.06	0.08
312	0.08	0.06	0	0.03	0.08	0.06	0.06	0.09
336[d]	0.06	0.05	0	0.02	0.05	0.04	0.06	0.08
360	0.04	0.03	0	0	0	0	0.03	0.06
384	0	0	0	0	0	0	0	0
408	0	0	0	0	0	0	0	0

[a] Milk from control cows and cows receiving 0.1 mg. chlortetracycline/lb body weight/day negative for chlortetracycline.

[b] Cow 777 removed from treatment on fifth day because of illness.

[c] Method sensitivity, 0.01 γ/ml.

[d] Chlortetracycline removed from the feed after the 14th day.

seen in all herds nor in all animals within a herd where gross evidence of this adjustment was apparent. The higher doses which were given early in the trial are not believed to have been responsible per se for the effect, because even higher doses have been fed to cattle with no apparent effect, and the same doses used for spot treatment in the experiments of Henderson (6) had no untoward results.

The feeding of 0.1 mg. of chlortetracycline per pound of body weight per cow per day produced no effect on feed consumption and only a very slight decrease in milk production, which was of short duration. This effect on production was not reported by Henderson *et al.* (7) and was reported only occasionally by others (10, 13) using chlortetracycline at this level in commercial dairy herds. It would appear that, as in beef cattle, ten times the recommended level is safe to feed but that even the recommended level may occasionally cause digestive upsets that are overcome within a few days.

Milk production paralleled feed consumption. Since the cows receiving 1.0 mg. of chlortetracycline per pound of body weight returned to normal production relatively quickly, failure of the group on the intermediate dosage schedule to return as quickly to full production was attributed to individual idiosyncrasy. All cows, including the controls, were producing less at the end of the experiment than at the beginning, probably because of the unusual weather conditions.

Milk samples were negative for chlortetracycline in the cows fed 0.1 mg. per pound of body weight per day. These results are in agreement with the work of Henderson *et al.* (7), cited earlier. Other workers who fed similar dosage levels of antibiotic did not report assay of the milk; therefore, no direct comparisons can be made with other studies.

The most common range of levels of antibiotic in the milk for the group fed 0.5 mg. per pound of body weight after correction of the dosage error was 0.02 γ/ml to 0.06 γ/ml, and this increased in direct proportion to the dosage at the higher treatment level. Chlortetracycline, fed at 0.5 mg. per pound of body weight, therefore produced only minimal levels in the milk. Loosli and Warner (11) reported no antibiotic in the milk when 700 mg. of chlortetracycline was fed daily for ten days. This is slightly more than 0.5 mg. per pound of body weight. Since the assay method used was not described, it is not known whether the technique was less sensitive or whether this was a question of individual idiosyncrasy. Henderson *et al.* (7) reported detectable amounts of antibiotic in the milk (without giving exact figures) at the higher dosage levels. Recent communication (6) indicates that the levels reported here are similar to those obtained in the earlier work (7).

Since Martin *et al.* (12) indicated that 0.32 mg. per pound of body weight did not show measurable amounts of chlortetracycline in the milk, 0.5 mg. per pound of body weight approaches the lowest feeding level that will show activity in the milk. As previously indicated, the Food and Drug Administration's approved feeding level is 0.1 mg. per pound of body weight per cow per day. This dose is safe for cattle and, because approximately five times the dose is required to produce a detectable amount of antibiotic in the milk, it is unlikely to affect the suitability of milk for cheese manufacture or consumption by sensitive individuals.

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FREEZING BOVINE SEMEN. V. PRACTICABILITY OF COLLECTING AND FREEZING A LARGE NUMBER OF SUCCESSIVE EJACULATES [1, 2]

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SUMMARY

From six to 37 successive ejaculates were collected during 12, 2-hr. depletion trials with eight dairy bulls, and used to investigate (a) the proportion of ejaculates acceptable for freezing and (b) the freezability of the acceptable ejaculates. Only ejaculates showing an initial motility of 30% or more and a minimum of 150 million motile spermatozoa were frozen. On this basis, the percentage of culled ejaculates increased as the number of consecutively collected ejaculates increased. Considering the first five consecutive ejaculates, only 6.7% were culled; among the first ten consecutive ejaculates, 11.6% were culled; whereas, among all 257 ejaculates collected, 35.8% were culled. There was no significant difference in the freezability of the acceptable ejaculates among the first five or first ten individual ejaculates, and freezability was comparable with that obtained with ejaculates collected from the same bulls when on a frequency of two or six ejaculates per week. Beyond the tenth ejaculate, freezability was generally, but not significantly, poorer and collection of more than ten consecutive ejaculates probably would be of no practical value. Pooling acceptable ejaculates from a depletion trial resulted in freezability comparable to freezing the ejaculates separately. With adequate sexual preparation, the collection of six or seven consecutive ejaculates from a given bull should make available large numbers of sperm at one time, which should freeze satisfactorily.

Preservation of bull spermatozoa by freezing has become a practical procedure in routine artificial breeding. In most associations, bulls are ejaculated once or twice every four to seven days. Thus, semen from these bulls must be frozen frequently in relatively small quantities. A considerable saving in both time and labor would result if bulls could be ejaculated a number of times consecutively at weekly or monthly intervals to provide larger quantities of semen for freezing. This would permit the establishment of a large bank of frozen semen from a particular bull very rapidly.

Several reports have shown that a greater percentage of spermatozoa survive freezing to −79° C. in second than in first ejaculates when they are collected consecutively (4, 6, 9, 10). Willett and Ohms (10) attributed the superiority in freezability of sperm from the second over the first ejaculate to unknown characteristics of the cells and not to differences in seminal plasma. VanDemark *et al.* (9) postulated that the maturity of the sperm might be a factor affecting freezability. Holt (3) and Madden (6) suggested that the higher concentration of sperm in the first ejaculate than in the second or third ejaculate may play an important role in contributing to poorer freezability of sperm in the first

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ejaculate. Willett and Ohms (10) and Kinney and VanDemark (4) reported progressive improvement in the freezability of consecutively collected ejaculates through the fourth, with a gradual decrease thereafter through the 18th to 20th, ejaculate.

This paper reports the results of a study to determine (a) the proportion of ejaculates acceptable for freezing when a large number of ejaculates were collected during 2-hr. depletion trials and (b) the freezability of the acceptable ejaculates.

EXPERIMENTAL PROCEDURE

Eight bulls of the Guernsey, Holstein, and Jersey breeds were allowed to ejaculate without restraint, false mounting, or encouragement during a 2-hr. depletion trial (1). Four of the bulls were subjected to a second 2-hr. depletion trial seven days following the first, so that a total of 12 different collection days (depletion trials) was evaluated (Table 1). A total of 257 ejaculates was obtained, or an average of 22 ejaculates per depletion trial (range six to 37).

Only those ejaculates having an initial motility of 30% or more and a minimum of 150 million motile spermatozoa were frozen. Each of the 165 ejaculates which met the above requirements was diluted as soon as possible after collection to 30 million motile sperm per milliliter in 5 ml. of heated fresh skimmilk and cooled to 5° C. in 2 to 3 hr. An equal volume of heated skimmilk containing 26% glycerol by volume and 2.5% fructose by weight was added to the diluted semen

TABLE 1

A comparison of the post-thawing motility immediately after freezing of ejaculates collected during depletion trials with that of ejaculates collected at a frequency of two or six ejaculates per week

Breed and bull	Age of bull	No. of ejaculates collected during the 2-hr. depletion	All ejaculates frozen		Ejaculates 1 through 10 frozen		Two or six ejaculates per week	
			No. of ejaculates	Av. % motility	No. of ejaculates	Av. % motility	No. of ejaculates	Av. % motility
	(yr.)							
G-1	2	7	6	42.5	6	42.5	4	35.0
G-2	2	9	8	52.8	8	52.8	12	45.6
G-3	7	11	10	56.5	10	56.5	11	50.7
G-3[a]		11	11	51.1	10	50.2		
Total and av.		38	35	51.6	34	51.4	27	46.1
H-1	4	6	5	44.5	5	44.5	14	50.9
H-2	2	29	13	34.8	6	40.4	2	40.0
H-3	5	30	28	37.8	10	41.0	9	46.9
H-3[a]		37	17	34.6	10	39.5		
Total and av.		102	63	36.9	31	40.9	25	48.6
J-1	6	37	18	25.8	4	21.4	7	28.9
J-1[a]		20	13	47.9	10	48.0		
J-2	6	31	21	44.3	10	41.8	10	37.5
J-2[a]		29	15	41.0	10	52.5		
Total and av.		117	67	39.3	34	42.1	17	34.0
Grand total and av.		257	165	41.0	99	44.8	69	44.0

[a] Bulls appearing twice were subjected to two depletion trials at a seven-day interval.

at 5° C. in five equal portions at 6-min. intervals. The final diluted semen contained 1,000 units each of penicillin and dihydrostreptomycin and 15×10^6 motile sperm per milliliter. During a 6-hr. equilibration period, 1.0-ml. portions of diluted semen were placed in 1.6-ml. glass ampules and sealed. The samples were frozen in an ethyl alcohol bath by gradual addition of dry ice to lower the temperature of the bath at the rate of 3° C. per minute from +5 to −15° C., 10° C. per minute from −15 to −50° C., and approximately 12 to 15° C. per minute from −50 to −79° C. In five of the depletion trials the ejaculates also were pooled and samples of the pooled semen were processed and frozen as above.

One of each pair of duplicate ampules was thawed in a 5° C. water bath immediately after freezing, while the other was thawed after ten days of storage at −79° C. Duplicate estimations of the percentages of motile sperm were made on each ampule and averaged for statistical analysis. The ampules were coded and randomly selected for thawing to reduce observer bias.

RESULTS AND DISCUSSION

Based on Table 1, 92, or 36%, of the 257 ejaculates were not acceptable for freezing. Fourteen of the 92 ejaculates had insufficient numbers of motile sperm, while 78 had less than 30% motile spermatozoa. Of the ejaculates with less than 30% motile spermatozoa, 62, or 80%, exhibited no motility. This is similar to the observation of Almquist and Hale (1), who noted that, except for an increase in the incidence of samples with no motility, the per cent of motile sperm was not appreciably altered by successive ejaculations. Considering only the first ten consecutive ejaculates (total of 112) collected in the present experiment, 13, or 12%, were undesirable for freezing. Of the first five consecutive ejaculates collected (total of 60) only four, or 6.7%, were not suitable for freezing.

Table 1 compares the average freezability of acceptable ejaculates in the 12 depletion trials with the average of those for the first ten consecutive ejaculates and the average of the two to 14 first or second ejaculates collected from the same bulls when they were on a collection frequency of two or six ejaculates per week (two ejaculates one day per week −2 ×; two ejaculates each on Monday, Wednesday, and Friday −6 ×). The differences among the three groups of ejaculates and among breeds were small. The average freezability of the ejaculates in the 1 to 10 category was slightly higher than the average of the ejaculates collected on frequencies of two or six per week, whereas the average for all samples collected and frozen in the 2-hr. depletions was slightly lower. The average freezability of ejaculates from the Holstein bulls was lower than that of the ejaculates collected at a frequency of two or six per week. The reverse was true for the Guernsey and Jersey bulls. However, final conclusions on breed differences can not be made because of the very small number of bulls in this study.

As shown in Table 1, the average freezability of acceptable ejaculates collected from three of the four bulls 1 wk. following a previous depletion trial was lower than in the first trial. Considering only the first ten ejaculates collected, however, this relationship was true for only two of the four bulls. Nevertheless, this indicates that for some bulls it may be desirable to allow more than 1 wk.

of rest between depletion trials for optimum freezability when ten or more consecutive ejaculates are collected.

The average semen characteristics for all the ejaculates frozen from the eight depletion trials at weekly intervals and from the first ten consecutive ejaculates in all depletion trials, as well as for those collected at a frequency of two or six ejaculates per week are presented (Table 2). With the four bulls subjected to two depletion trials, the average number of sperm per ejaculate in each trial was the same. Semen volume per ejaculate was lower in the second trial, but was offset by an increase in concentration. There was no effect on percentage of motile sperm. These data do not explain the decreased freezability of the ejaculates in the second depletion trial. The average volume and concentration of the acceptable ejaculates among the first ten consecutive ejaculates in all 12 depletion trials was significantly lower than for ejaculates collected at frequencies of two or six weekly. The pattern of decline in semen characteristics is similar to that presented by Almquist and Hale (1).

Table 3 summarizes in increments of five the freezability of the 165 acceptable ejaculates and the percentage of the 257 consecutively collected ejaculates which were suitable for freezing. There was a progressive decline both in the proportion of suitable ejaculates and in the freezability among ejaculate groups. The largest decrease in freezability occurred between the 11-15 and 16-20 ejaculate groups. The decrease in post-thawing motility accompanied the decrease in the percentage of motile sperm prior to freezing. These results agree with those of VanDemark *et al.* (9). In the present experiment, the average percentage of motile sperm for the ejaculates frozen from the first ten consecutive ejaculates (total of 99 ejaculates frozen) when thawed immediately after freezing was 44.8%; whereas, the average for those frozen from ejaculates 11 through 37 (total of 66 ejaculates

TABLE 2

Comparison of semen characteristics for all ejaculates frozen from depletion trials at weekly intervals and of ejaculates frozen from the first ten consecutive ejaculates in 12 depletion trials with those collected at a frequency of two or six ejaculates per week

Acceptable ejaculates from two successive depletion trials at a seven-day interval from four bulls			
	1st depletion trial	2nd depletion trial	Difference
Total number of ejaculates collected	109	97	12
Total number of ejaculates frozen	77	56	21
Volume of semen per ejaculate *(ml.)*	3.31	2.33	0.98
Motile sperm *(%)*	59.9	60.5	0.6
Number of motile sperm per milliliter semen *($\times 10^6$)*	535	802	267
Number of sperm per ejaculate *($\times 10^6$)*	1,833	1,806	27
Acceptable ejaculates from the first ten collected in 12 depletion trials			
	Depletion trials	2 or 6 ejaculates per week	Difference
Total number of ejaculates collected	112	.....	.....
Total number of ejaculates frozen	99	69	.....
Volume of semen per ejaculate *(ml.)*	3.36	4.09	0.73[a]
Motile sperm *(%)*	62.0	64.3	2.3
Number of sperm per milliter semen *($\times 10^6$)*	887	1,398	521[b]

[a] $P < 0.05$.
[b] $P < 0.01$.

TABLE 3

Comparison of the acceptability and freezability of ejaculates collected during a 2-hr. depletion trial

(Weighted average for 12 collections from eight bulls)

Ejaculate group	No. of ejaculates collected	No. of ejaculates frozen	Per cent of ejaculates frozen	Pre-freezing motility	Post-thawing motility after storage at −79° C. for		
					0 day	10 days	Av.
				(%)	(%)	(%)	(%)
1–5	60	56	93.3	56.5	45.4	37.0	41.2
6–10	52	43	82.7	56.0	44.1	33.7	39.0
11–15	37	24	64.9	54.8	38.5	30.9	34.7
16–20	35	18	51.4	49.2	34.6	21.2	28.1
21–25	30	15	50.0	51.0	32.0	19.5	25.9
26 and over	43	9	20.9	52.2	33.3	20.0	27.1
Total and av.	257	165	64.2	54.6	41.0	31.2	36.1

frozen) was only 38.3%. In addition, 54.5% of ejaculates 11 through 37 (total of 145 ejaculates collected) were unsatisfactory for freezing, whereas only 11.6% of ejaculates 1 through 10 were not satisfactory. Therefore, it appears that it would not be desirable to collect more than ten consecutive ejaculates from dairy bulls for freezing and storage.

Since the previous results indicated that it probably would be undesirable to collect more than ten consecutive ejaculates from a bull for freezing, the data were analyzed on the basis of the first five and the first ten consecutive ejaculates.

TABLE 4

Freezability of five and ten ejaculates collected in succession from dairy bulls

Ejaculate number	Prefreezing motility	Post-thawing motility after storage at −79° C. for		
		0 day	10 days	Av.
	(%)	(%)	(%)	(%)
	Average of 11 depletion trials from eight bulls[a]			
1	59.1	42.0	31.4	36.7
2	54.1	48.0	39.8	43.9
3	60.9	48.0	40.4	44.2
4	60.4	48.0	40.4	44.2
5	59.1	50.9	42.5	46.7
Av.	58.7	47.4	38.9	43.1
	Average of seven depletion trials from four bulls[a]			
1	59.3	45.4	32.5	38.9
2	52.9	48.2	36.4	42.3
3	62.9	50.3	40.0	45.2
4	60.0	50.0	37.5	43.8
5	57.9	50.7	38.9	44.8
6	58.6	48.2	32.5	40.4
7	59.3	50.7	37.9	44.3
8	57.1	44.6	34.3	39.5
9	55.0	42.5	34.3	38.4
10	60.7	41.1	30.0	35.5
Av.	58.4	47.2	35.4	41.3

[a] Includes only depletion trials in which the first five or the first ten ejaculates were acceptable for freezing.

No ejaculates were discarded. Data presented in Table 4 show the freezability of the first five ejaculates for 11 trials and of the first ten for seven of the 12 trials. The remaining trials were excluded because one or more ejaculates did not meet the minimum standards or ten ejaculates were not obtained. In both analyses of variance no significant differences among individual ejaculates were found when tested with the respective highly significant bull × ejaculate interaction. Although there was a highly significant ($P < 0.01$) decrease in viable sperm after ten days of storage at −79° C., the interaction between ejaculates and length of storage at −79° C. was not significant. The interaction between bulls and loss of viable sperm during storage at −79° C. was highly significant ($P < 0.01$) and this agrees with other reports (4, 6, 7). The freezability of consecutive ejaculates increased through the fifth to seventh ejaculate, with a slight decrease thereafter through the tenth ejaculate. However, the decrease was not of sufficient magnitude to warrant culling any of the first ten ejaculates.

Table 4 also shows a difference in freezability in favor of all but the ninth and tenth ejaculates over the first ejaculate. The superiority of the second ejaculate over the first agrees with other studies (4, 9, 10). In the present experiment, the second ejaculate froze better ($P < 0.05$) and exhibited less loss of motile sperm after ten days of storage at −79° C. ($P < 0.01$) than did the first. Although perhaps influenced by the lack of special sexual preparation prior to collection, the second ejaculate was higher in volume, fructose content, and initial motility, but was lower in sperm concentration ($P < 0.05$), total sperm, and total motile sperm. There was no difference in rate of motility or per cent of live sperm as determined by differential staining.

Table 5 compares the freezability of the pooled ejaculates from five of the depletion trials with the average freezability of the same ejaculates individually. In general, post-thawing motility of the pooled semen was slightly, but not significantly, higher than the average of the individual ejaculates. Pooling consecutive ejaculates would greatly simplify the processing of semen for freezing.

Based on the average ejaculate characteristics shown in Table 2, a total of

TABLE 5

Effect of pooling acceptable ejaculates on the post-thawing motility of spermatozoa

Bull	Prefreezing motility			Post-thawing motility after storage at 79° C.					
				Av. of individual ejaculates			Pooled ejaculates		
	No. of ejaculates frozen	Av. of individual ejaculates	Pooled ejaculates	0 day	10 days	Av.	0 day	10 days	Av.
		(%)	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
G–3[a]	11	61.4	65.0	51.1	31.8	41.5	57.5	42.5	50.0
J–1	18	37.8	40.0	25.8	15.4	20.6	32.5	17.5	25.0
J–1[a]	13	58.8	50.0	47.9	40.0	43.9	45.0	45.0	45.0
J–2	21	55.0	50.0	44.3	39.5	41.9	40.0	30.0	35.0
J–2[a]	15	51.7	55.0	41.0	33.3	37.2	52.5	50.0	51.2
Total and av.	78	52.9	52.0	42.0	32.0	37.0	45.5	37.0	41.2

[a] These trials were conducted seven days after a previous depletion trial.

1,232 ampules of semen containing 15×10^6 motile sperm per ampule could be frozen from ten consecutive ejaculates. Only 490 ampules could be frozen on a collection frequency of two ejaculates a week. Thus, approximately 2.5 times as many ampules of semen could be frozen at weekly intervals by collecting ten consecutive ejaculates a week, rather than two ejaculates a week. In addition, the higher frequency of ejaculation should save time in processing and freezing semen. Other studies at this laboratory (2) show that with more intensive sexual preparation than used in the present study, larger sperm numbers can be collected at frequencies of two or six ejaculates per week. With adequate preparation for each ejaculate, the majority of sperm available for ejaculation should be obtained by collecting six or seven ejaculates rather than ten or more at weekly intervals without preparation. Fertility trials are needed to substantiate the generally favorable laboratory results.

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INFLUENCE OF PROLONGED INCUBATION OF BULL SEMEN AT 37° C. ON FRUCTOLYSIS, LACTIC ACID, AND MOTILITY [1]

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SUMMARY

Eighteen semen samples, three from each of six dairy bulls, were concentrated by centrifuging and removing supernatant plasma to one-half of the original volume. The sperm and remaining plasma were extended 1:6 with 2.9% Na-citrate and divided into 20 tubes of 1 ml. and incubated at 37° C. Duplicate tubes were removed at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 hr. of incubation, for determination of pH, fructose, lactic acid, and motility rating on a 0 to 10 scale (10 = 100%). At 0 hr. of incubation the averages were: (a) pH—6.87; (b) fructose—4.27 ± 0.33 mg/ml of semen; (c) lactic acid—0.84 ± 0.10 mg/ml of semen, and (d) motility—6.56 ± 0.89. pH remained essentially unchanged during incubation. Fructose utilization by 10^8 sperm/ml/hr was 2.02 ± 0.18 mg. during the first 0.5 hr. of incubation. Motility ceased when fructose concentration was less than 0.06 mg/ml of extended semen and was seriously impaired at observed levels of 0.14 to 0.29 mg/ml. Lactic acid accumulation reached levels of 0.6 mg/ml of extended semen, but was not a principal cause of reduced motility. It is recommended that fructose levels in metabolism studies be expressed as mg/ml of extended semen, as well as the concentration per milliliter of whole semen, since different extension rates are frequently used.

Lardy and Phillips (12) recognized that glycolysis was the primary source of energy for motility of bull sperm. Following the identification of fructose as the principal sugar in semen (13) many studies, as earlier reviewed (3, 5, 6), have shown that bull sperm metabolize fructose at variable rates, depending on motility, sperm cell concentration, extent of dilution, incubation time and temperature, and amount of fructose initially present in the incubate.

Hopwood *et al.* (11) first recognized that removal of fructose by sperm fitted, in an empirical way, a unimolecular (first-order) reaction. These workers presented a method for expressing fructolysis rates independent of sperm concentration. Later, Mixner *et al.* (14) also showed that fructose utilization rate by incubating sperm followed essentially the first-order reaction, and derived a method for expressing fructolysis rate independently of initial levels of fructose present. Freund *et al.* (10) investigated the problem further and concluded that, for comparative purposes, fructolysis rates should be expressed independently of sperm concentration and levels of fructose initially present.

Lactic acid accumulates in an incubating sperm-substrate mixture when a sugar is metabolized. However, sperm are capable of using lactic acid further; under a variety of experimental conditions from 9 to 75% of the fructose loss has not been accounted for as lactic acid. Like fructolysis rate, lactic acid gain during incubation shows a highly significant correlation with initial motility and

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motility at the end of the incubation period (9). Fructolysis rates by 10^9 sperm/ml and lactic acid gain (5, 6, 9) have been positively correlated with non-return rates on a between-bull basis. High lactic acid accumulations have been positively associated with maintenance of motility under conditions of incubation (6, 9) and storage (16).

Maintenance of motility during semen storage at 5° C. remains the single most practical test of fertilizing capacity. Thus, it appeared important to examine the relationship between sperm motility and incubating substrate levels of fructose and lactic acid. It was the purpose of this experiment to investigate the substrate levels of fructose required to support progressive motility. Lactic acid accumulation was also followed, to determine if lactic acid is metabolized at a greater rate when substrate fructose is very low.

EXPERIMENTAL PROCEDURE

Eighteen semen samples were collected from six mature bulls in the State College of Washington dairy herd. Three samples, pooling first and second ejaculates, were collected from each bull at approximately weekly intervals. Immediately after collection, 6 ml. of semen was placed into a 50-ml. pyrex conical tube and centrifuged at 750 g for 8 min. After centrifugation, 3 ml. of supernatant plasma was removed to increase the sperm : plasma ratio so that seminal fructose would be more completely utilized by 6 hr. of incubation. This procedure was believed satisfactory because Albright *et al.* (1) have shown that centrifuged twice-washed sperm, resuspended in milk, maintain motility as well as centrifuged sperm resuspended in the original plasma. Motility regained immediately following centrifuging was essentially the same as before. The separated sperm and remaining plasma were extended 1 : 6 with 2.9% Na-citrate containing 1 mg. of streptomycin/ml of extended semen. The extender was forcibly ejected into the compressed sperm cells. One milliliter of the semen-extender mixture was placed into 15-ml. centrifuge tubes for incubation in a water bath at 37° C. Subsequent samplings were in duplicate for determination of motility, fructose, and lactic acid. Samplings were made at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 hr. of incubation. Immediately after determining motility, 5 ml. of cold (5° C.) 10% trichloroacetic acid were added to duplicate tubes after each interval of time, then thoroughly mixed and placed in an ice water bath. After approximately 30 min., the samples were centrifuged, filtered, and stored in a refrigerator for fructose and lactic acid determinations the following day.

Motility was subjectively rated on the basis of progressively motile sperm on a 0 to 10 scale where 10 was estimated as 100%. Low-power microscopy was used. Sperm concentrations were determined in duplicate with a hemocytometer. Fructose was measured by the method of Roe (15), as modified and used by Erb *et al.* (5). Lactic acid was determined by the method of Barker and Summerson (2), as modified by Ehlers and Erb (3). The colorimetric determinations for fructose and lactic acid were with an Evelyn colorimeter. The pH was determined with a Beckman glass-electrode pH meter. Statistical procedures were as outlined by Snedecor (17).

RESULTS AND DISCUSSION

The 18 extended semen samples at the start of incubation averaged as follows: (a) Motility—6.56 ± 0.89; (b) 10^9 sperm/ml—2.11 ± 0.14; (c) lactic acid—0.84 ± 0.10 mg/ml, and (d) fructose—4.27 ± 0.33 mg/ml. Figure 1 shows that fructose use by 10^9 sperm/ml/hr declined from an acceptable rate of 2.02 ± 0.18 mg. during the first 30 min. of incubation to below a 0.5 mg. rate between 1.0 and 1.5 hr. Motility averaged less than 1.0 by 3 hr. of incubation. Lactic acid reached near maximum concentration by 1.5 hr. of incubation and this coincided with less than 1.0 mg. of fructose per milliliter of semen (only 0.14 mg/ml of incubate). The pH declined very little, but the rate of decline was greatest during the early part of incubation. An extra tube containing approximately 1 ml. of extended semen was available for each sample and was incubated along with the original 20. After motility ceased, and in some cases 1 to 2 hr. later, addition of one or two drops of a fructose solution or 0.5 ml. of seminal plasma to these nonmotile samples resulted in revived motility. The degree of revival was, in general, related to time after motility ceased. If fructose or seminal plasma was added soon after no progressive motility was observed, sperm movement increased to approximately that observed at the start of incubation. This is interpreted to

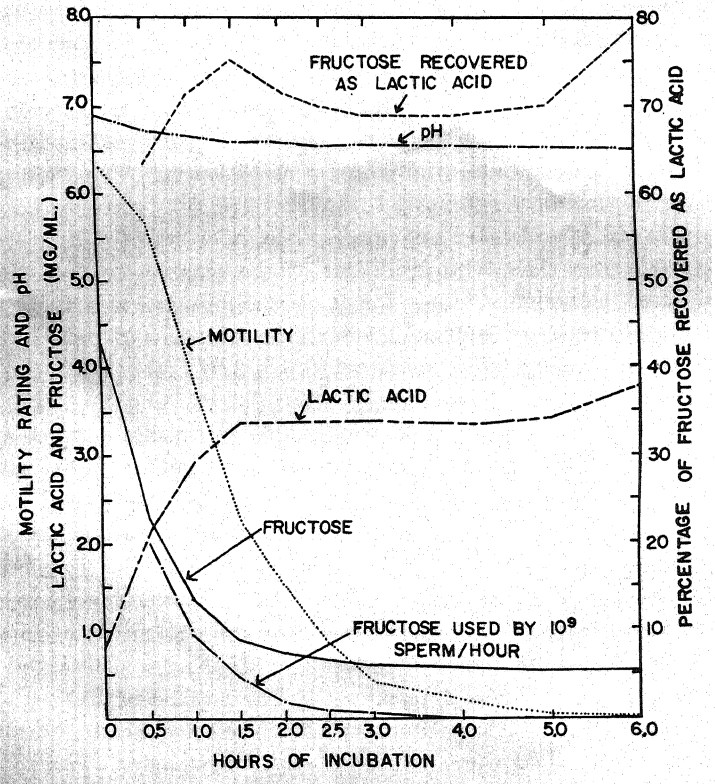


FIG. 1. Changes in motility, fructose, lactic acid, fructose utilization by 10^9 sperm/hr, fructose recovered as lactic acid, and pH during 6 hr. of incubation at 37° C.

mean that under the conditions of this study lactic acid accumulation was not great enough to reduce motility.

Erb *et al.* (5) estimated that fructose in excess of 1 mg/ml of semen was necessary to maintain a reasonable utilization rate by 10^9 sperm. Because the extension rate was 1 : 4, the fructose level per milliliter of incubate was one-fifth as high, or 0.2 mg/ml of incubate. In this study, the semen was extended 1 : 6. Figure 1 shows 1.35 mg/ml of fructose after 1 hr. and the concentration per milliliter of incubate was one-seventh, or 0.19 mg/ml. The motility at this time averaged 3.7 as compared with 6.6 for the earlier study (6). The data in Figure 1 indicate that fructose concentrations of approximately 0.15 mg/ml of incubate (one-seventh the values in Figure 1, to compensate for extension) are at the critical level, as evidenced by the rapid decline in progressive motility and the small increases in lactic acid. The correlation, using covariance, between fructose (mg/ml) and motility was 0.68 for all samples in this study and 0.66 for samples within time of incubation and bull. The correlation between fructose use/hour and motility was 0.85 for all samples and 0.41 for samples within time of incubation and bull. This latter value corresponds with a sample within-bull correlation of 0.38 in an earlier study (6).

In 16 of the 18 samples, the available fructose was nearly all used before motility rated less than two. The remaining two semen samples were from different bulls. The fructose initially present was, respectively, 4.1 and 5.7 mg/ml of semen and the utilization rate/hour was above average for the first hour. Both samples were below average in sperm concentration. Motility was maintained longer than average. Lactic acid accumulation proceeded normally. Since stress caused by low fructose was not created in these two samples, they were omitted from the summary shown in Figure 2.

The data for 16 samples exhausting fructose in 6 hr. or less of incubation were summarized by motility, independently of time of incubation. Concentrations of fructose and lactic acid are shown in Figure 2 on a basis of mg/ml of extended semen, instead of the usual mg/ml of semen (Figure 1). Figure 2 shows that lactic acid concentrations were maximum (0.61 mg/ml) for motility ratings of 2.

There is little possibility that concentrations at this level inhibit motility because in a previous study (9) motility after incubation for 1 hr. was highest for samples exceeding 0.6 mg. of lactic acid/milliliter of semen. Figure 2 also shows that motility essentially ceases when fructose concentration is less than 0.06 to 0.08 mg/ml of extended incubating semen. These values were adjusted for sperm cell volume (6, 8); thus, the range of 0.06 to 0.08 mg/ml is higher than if adjustment had not been made.

From Figure 1 it can be calculated that motility declined 10% and fructose declined 45% during the first 0.5 hr. of incubation. Fructose decreased to 0.34 mg/ml of extended semen at this time. Motility declined 42% and fructose 68% during the first 1 hr. of incubation, and fructose decreased to 0.18 mg/ml of extended semen. This suggests that the level of fructose required for good motility is between 0.34 and 0.18 mg/ml of extended semen. From Figure 2 it

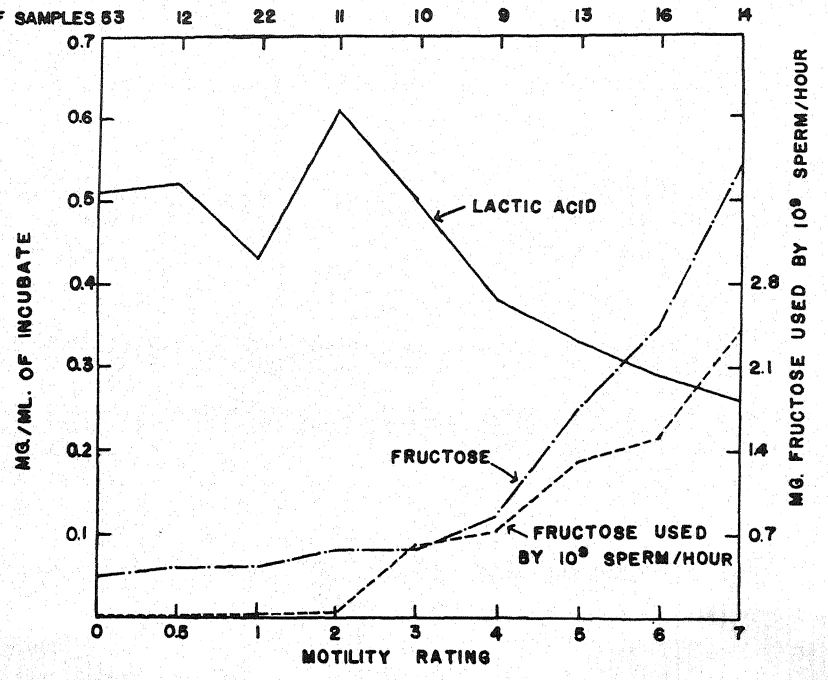


FIG. 2. The relationship between motility rating and lactic acid, fructose, and fructose utilization by 10^9 sperm/ml/hr.

appears that approximately 0.25 mg. of fructose per milliliter of incubating semen-extender mixture is required to prevent excessive decline in average motility. Since the semen samples varied in initial motility, a motility rating of five would represent a sizeable drop in one sample and none in another. A clearer picture was obtained by determining the average fructose at the incubation time preceding a drop of at least one in motility rating and comparing with average fructose at the time a drop of this magnitude was observed. Fructose declined 50% or to an average of 0.29 mg/ml of extended semen before a noticeable decline in motility (average of 6%) occurred. During the next 0.5 hr., motility rating was 4.8, a decline of 22%, and fructose was down an additional 27%, or to an average of 0.14 mg/ml of extended semen. Figure 2 describes the changes quite well for motility ratings of less than five. Thus, the critical level of fructose for good motility under the conditions of this experiment was less than 0.29 mg/ml of extended semen and more than 0.14 mg/ml and probably not far from 0.25 mg/ml. This coincides with 1.25 mg/ml of semen when extended 1:4 prior to incubation (5) and 1.75 mg/ml when extended 1:6 (Figure 1).

General discussion. Ehlers and Erb (3) have observed that whole semen–extender volume relationships (phosphate) are of greater importance than actual sperm concentration. Fructose utilization and lactic acid production were similar through an extension range of 1:1 to 1:8, but declined at 1:16 and 1:24. Based on the fructose initially present, the 1:8 extension left an average of 0.68 mg.

of fructose per milliliter of incubate and a 1:16 extension averaged 0.32 mg/ml (3). The utilization of fructose by 10^9 sperm for the first hour of incubation was 1.4 mg. The motility averaged 6.5 (3). This corresponds with 0.34 mg/ml of fructose and 1.4 mg/hr for a motility rating of six in Figure 2. Milk has been shown to provide energy for maintenance of motility (1). Therefore, it is significant that when Ehlers and Erb (3) extended semen 1:24 with milk, lactic acid production was slightly greater than when extended at 1:4. Purposely creating conditions to insure exhaustion of fructose failed to show that an appreciable extra amount of lactic acid was used. Instead, motility declined rapidly when fructose in the incubating semen was below approximately 0.1 mg/ml.

This study shows that fructose in the absence of other glycolyzable sugars is necessary for maintenance of motility and that 0.14 to 0.29 mg/ml of incubate is required to maintain average motility. Additional fructose or seminal plasma revived motility in semen incubates after initial rates had declined to zero. This shows that lactic acid levels (maximum reached was 0.6 mg/ml of incubate) were not high enough to inhibit motility. Also, energy to maintain the living sperm cell may be entirely from nonglycolytic sources (12), because motility was markedly revived even after 1-2 hr. in the nonmotile state at 37° C. The revival of motility to approximately initial levels was probably higher than would have been the case if no seminal plasma had been removed. In reality, slightly over one-half of the fructose initially present was removed in the seminal plasma, because the sperm cells contain little or no fructose. Under these conditions, none of the intracellular reserves, and probably very little of the substances normally bound to the peripheral surfaces of the sperm cell, were removed. Therefore, the ratio of intra- and extra-cellular nutrient supply was altered considerably. The results imply that the well-being of nonmotile sperm is not impaired if sufficient energy from nonglycolyzable sources is present.

Progressive motility as a practical test of semen quality serves indirectly as a measure of metabolic activity initially and during storage (6). More consideration should be given to expressing fructose concentration in mg/ml of extended semen, rather than on an amount in the unextended semen. Little or no progressive motility can be expected when fructose is below 0.06 mg/ml of extended semen.

This study is not in disagreement with principles set forth concerning the relationship between level of initial fructose and sperm concentration and the fructose utilization rate during incubation (5, 10, 11, 14). Consideration of motility would assist in distinguishing between sperm (semen samples) incapable of utilizing the sugar present and those capable of further sugar use when an exogenous source of energy is supplied in the extender.

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A STUDY OF BOVINE FOLLICULAR FLUID AND ITS EFFECT ON OXYGEN CONSUMPTION BY BOVINE SPERMATOZOA[1]

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SUMMARY

Five semen samples were obtained from each of two bulls and used to determine the effects of follicular fluid on oxygen consumption by washed spermatozoa. The spermatozoa in follicular fluid utilized 4.6 times more oxygen than did an equal number of spermatozoa in saline. It was found that 36.6% of this increase was due to glucose, 26.1% to protein; and the remaining 37.3% was apparently due to other unidentified, heat-stable, nondialyzable substances.

The pH of follicular fluid increased from about 7.4 to about 8.9 during incubation in Warburg flasks for 3 hr. at 37° C. This rise in pH was found to be due to the diffusion of carbon dioxide out of the fluid and its absorption by KOH in the center wells of the flasks. Fresh follicular fluid contained about 0.498 g. of carbon dioxide per liter.

Follicular fluid is thought to function in the nourishment, protection, and transportation of ova while they are being ovulated and carried into the oviduct. If this is true, it is very likely that spermatozoa also come into contact with follicular fluid at or near the time of fertilization. It has been reported that follicular fluid exerts a stimulating action on sperm motility (1,4) fructolysis and glycolysis (2, 3), and on respiration (3). Lutwak-Mann (2) concluded that the effect of follicular fluid on sperm fructolysis was due to its glucose content. However, preliminary work by Olds and VanDemark (3) indicated that the stimulating action of follicular fluid on sperm respiration was due to nondialyzable substances.

The present study was undertaken in an effort to learn more about the nature of bovine follicular fluid and how it affects the metabolic activity of bovine spermatozoa.

METHODS

Follicular fluid was collected from ovarian follicles of cows being slaughtered by a local meat-packing company. Composite samples of the fluid were stored at −10° C. until used. The following preparations of follicular fluid were made and used as diluents for washed spermatozoa: (1) Unaltered follicular fluid, (2) follicular fluid which had been dialyzed against water to remove salts and small molecules and then against physiological saline to reconstitute normal osmotic pressure, (3) follicular fluid which had been deproteinized by saturation with ammonium sulfate; the supernatant was dialyzed against water to remove the ammonium sulfate and then against saline to adjust the osmotic pressure; the protein precipitate was resuspended to the original volume and then dialyzed against water followed by physiological saline, (4) follicular fluid was heated to 55° C. for 30 min., for the purpose of inactivating enzymes.

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Five ejaculates were collected from each of two Jersey bulls at weekly intervals. The spermatozoa were washed three times in physiological saline (total of four centrifugations) and adjusted to a concentration of 1×10^9 cells per milliliter.

Oxygen uptake was determined by placing 0.2 ml. of the sperm suspension and 0.8 ml. of diluent in a Warburg flask and incubating for 3 hr. at 37° C. At the end of the incubation period, samples were removed from each flask for determination of pH and for microscopic examination.

Results and Discussion

The average oxygen uptake by bovine spermatozoa in the various media is shown in Figure 1. It may be observed that oxygen uptakes over the 3-hr. period were very similar for spermatozoa in whole follicular fluid, heated follicular fluid, and in dialyzed follicular fluid to which glucose had been added. There were no statistically significant differences among these three fluids. However, dialyzed follicular fluid produced significantly lower oxygen uptakes than any of the three aforementioned fluids. Likewise, there was no significant difference between uptakes produced by the solution containing follicular fluid protein and the dialyzed supernatant after protein precipitation. However, spermatozoa in both of these fluids produced significantly higher uptakes than did the spermatozoa in the physiological saline control.

These observations suggest that glucose metabolism accounts for a sizable portion of the oxygen consumption by spermatozoa in follicular fluid. However, the data also suggest that follicular fluid protein contributes to the oxygen consumption in some way other than through its enzyme content. Since the dialyzed supernatant fluid remaining after protein precipitation also produced higher uptakes than the saline control, it appears that follicular fluid contains a heat-

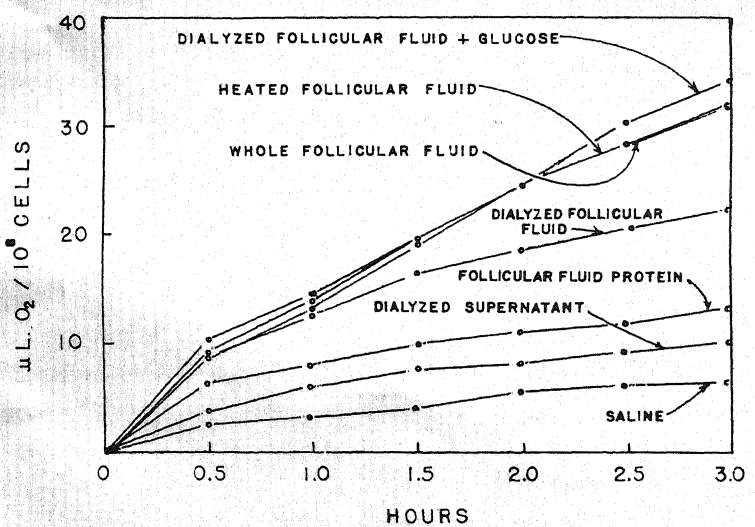


Fig. 1. Average oxygen uptake by 10^8 washed spermatozoa in various media.

stable nonprotein substance of large molecular size which contributes to oxygen uptake by spermatozoa. One might expect the effects of follicular fluid protein and of the dialyzed supernatant to add up to the effect of dialyzed whole follicular fluid. Actually, the sum of these two fell somewhat short of accounting for the effects of dialyzed follicular fluid. Therefore, it appears that ammonium sulfate treatment may have had an adverse effect which persisted on some follicular fluid component even after removal of the ammonium sulfate by dialysis. These data are summarized in Table 1.

Examination of Table 1 reveals that glucose is apparently the primary component of follicular fluid which is removed by dialysis, and it accounts for about 37% of the oxygen consumption by spermatozoa in whole follicular fluid. The protein of follicular fluid appears to account for about 26% of the oxygen consumption in whole follicular fluid. The remaining 37% appears to be attributable to a heat-stable, nonprotein, nondialyzable substance and also to a substance whose effectiveness is destroyed by the action of ammonium sulfate.

TABLE 1

Average oxygen uptake by 2×10^8 washed spermatozoa from ten samples of semen during three hours' incubation at 37° C. in saline and in various preparations of follicular fluid

Diluting media	Oxygen uptake	Increase over saline
	(μl.)	(μl.)
Unaltered follicular fluid	63.2	49.6
Heated follicular fluid	64.0	50.4
Dialyzed follicular fluid + glucose	69.9	56.3
Dialyzed follicular fluid	45.1	31.5
Saline solution of follicular fluid protein	26.5	12.9
Supernatant of f.f. after protein precipitation	23.3	9.7
Saline	13.6	0.0

The pH of the various fluids was determined before and after the 3-hr. incubation periods. While all of the fluids were near neutrality at the beginning of incubation, it was observed that whole follicular fluid samples (whether heated or unheated) consistently acquired pH's of about 8.85 during the incubation period; whereas, all of the samples prepared by treatments involving dialysis had an average pH of about 6.9 following incubation. At first, it was thought that perhaps the increase in alkalinity in whole follicular fluid was due to the metabolic activity of spermatozoa. However, incubation of whole follicular fluid without spermatozoa revealed that the pH rose to about 9.3 after 3 hr. in the Warburg flask. It was then concluded that the increase in alkalinity might be due to an escape of CO_2 from the solution. This idea was further substantiated when it was found that alkalinity did not increase when KOH was left out of the center well (KOH absorbs CO_2). Finally, the amount of CO_2 which could be driven off from follicular fluid by acid treatment was estimated with the Warburg apparatus. It was found that follicular fluid contained about 0.498 g. of CO_2 per liter. Calculations indicated that this amount, if allowed to escape from follicular fluid, could very likely explain the pH changes.

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EFFECTS OF GLYCEROL AND FRUCTOSE ON LIVABILITY, MOTILITY, AND ANAEROBIC GAS PRODUCTION OF BOVINE SPERMATOZOA[1]

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SUMMARY

In vitro manometric determinations of gas production by bovine spermatozoa under anaerobic conditions indicated a slight but statistically nonsignificant inhibitory effect of glycerol. The inclusion of 1% fructose with 1.0, 3.3, or 10.0% glycerol caused an immediate and marked stimulation of gas production by the spermatozoa.

Glycerol plus fructose maintained a greater degree of motility and livability of bovine spermatozoa at the end of 105 min. of incubation at 38° C. than did either fructose or glycerol alone. Under the conditions of this study, 1% glycerol plus 1% fructose was the apparent optimum for maintenance of spermatozoan motility and livability.

In 1949, Polge *et al.* (23) discovered that glycerol, a trihydroxy alcohol, would protect fowl spermatozoa during freezing and thawing. Since then, the mode of action (11, 12, 14, 25) and rate of entry (10, 13) of glycerol have been studied for several types of tissues. Although the practical aspects of freezing bovine spermatozoa have received considerable attention, relatively little work has been done on possible localization and mode of action of glycerol in these cells. Proof of entry has been presented largely by metabolic studies. Mann and White (16) found that glycerol was utilized aerobically but not anaerobically by ram spermatozoa. O'Dell *et al.* (21) observed that glycerol-1-C^{14} was utilized anaerobically by bovine spermatozoa and converted, in part, to radioactive carbon dioxide. White *et al.* (27) found that glycerol, under the conditions of their study, increased the oxygen consumption of bovine spermatozoa. Pickett and Merilan (22) employed an autoradiographic technique in an attempt to determine the loci of glycerol action and found glycerol-1-C^{14} to be associated with all parts of the spermatozoa. However, more of the C^{14} appeared to be with the nuclear area of the head than with the other parts.

The investigations of Mann (15) have shown fructose to be the normal glycolyzable carbohydrate present in bovine semen and that fructose is broken down, in part at least, to lactic acid. It has been further found that the rate of glycolysis is greater in nitrogen than in air (2, 3, 15). This study was conducted in an attempt to provide additional information concerning the metabolism of various concentrations of glycerol and glycerol-fructose combinations under anaerobic conditions.

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Materials and Methods

Bull semen was collected by means of an artificial vagina from five bulls maintained as part of the University of Missouri Agricultural Experiment Station Dairy Herd. Semen motility was rated according to the method of Herman and Madden (8) and only samples of four motility or better were used in the experiments. The spermatozoa were washed in calcium-free Krebs-Ringer phosphate (KRP), prepared according to Umbreit *et al.* (26), by suspending the semen in 167.0% of its original volume, centrifuging for 10 min. at 600 g, and removing the same amount of material that was added. Sperm cell concentration for each washed suspension was determined by the hemocytometer method (8).

Anaerobic metabolism of the spermatozoa was measured by gas production from the substrate, with KRP as the buffer. The measurements were made at 38° C. in a rectangular constant-volume Warburg Respirometer accommodating 14 reaction vessels, and adjusted to a shaking speed of 114 strokes per minute. To each of the flasks was added 2.0 ml. KRP, 0.5 ml. of the washed sperm suspension, and 0.5 ml. of the substrate (glycerol, fructose, or glycerol-fructose) was placed in the side arm. Final volume in each flask was 3.0 ml. The flasks were gassed with a mixture of 95.0% nitrogen and 5.0% carbon dioxide, using a manifold gassing arrangement.

After placing the flasks in the 38° C. water bath, they were allowed to equilibrate for 10 min. Following equilibration, readings were taken every 5 min. for 30 min., to establish a base. The side arms were then tipped and readings taken at 5-min. intervals for 75 min.

At the end of the experimental run, motility and per cent alive were determined on each flask. The per cent alive was calculated as the average of duplicate determinations made according to the method proposed by Mayer *et al.* (17).

Results and Discussion

The data presented in Figure 1 indicate that gas production by bovine spermatozoa was slightly inhibited (not statistically significant at 5% level) by all concentrations of glycerol studied. The control flasks contained some residual fructose, since the washing procedure used in these studies was not designed to remove all the fructose present in the seminal plasma but merely to dilute it while keeping the spermatozoan concentration high. Therefore, the controls in this study probably utilized the remaining fructose, whereas in the flasks containing glycerol, the glycerol in some way interfered with fructose utilization. However, the inhibition was not directly proportional to the final concentration of glycerol present. In the flasks containing 8.3 and 10.0% glycerol, most of the inhibition occurred during the first 15–30 min. after tipping; whereas, at lower glycerol concentrations, less inhibition occurred in the same time interval. This indicated that perhaps glycerol concentrations of 7–10% should be added stepwise, as done by some investigators (1, 5, 6, 20, 24).

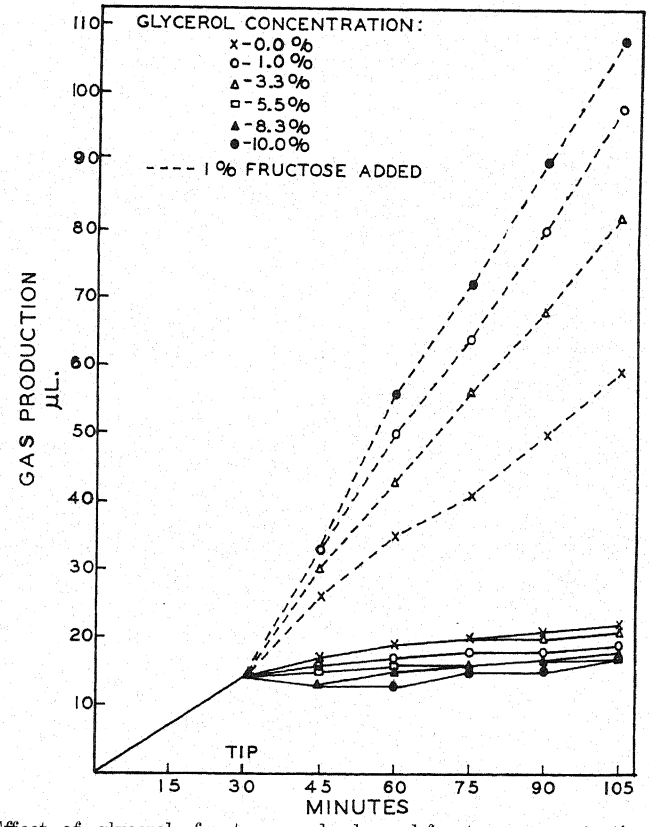


FIG. 1. Effect of glycerol, fructose, and glycerol-fructose concentrations on anaerobic metabolism of bovine spermatozoa (1.57×10^7 cells/ml) suspended in calcium-free Krebs-Ringer phosphate. Average for split-sample determinations with 15 individual semen ejaculates.

The addition of 1.0% fructose to the flasks, at the same time 1.0, 3.3, and 10.0% glycerol were added, caused an immediate and marked stimulation of gas production. However, these results do not show whether the increased gas production was the result of the utilization of fructose or glycerol, or both. In view of some recent investigations, it appears probable that the carbon dioxide was being produced from both glycerol and fructose. Mann (15) and Moore (19) have shown that glucose and fructose appear to be utilized through essentially identical pathways. Flipse and Almquist (4) have shown that lactic acid produced from glucose-C^{14} by bovine spermatozoa was, in part, broken down to carbon dioxide. O'Dell *et al.* (21) found that glycerol-1-C^{14} was utilized by bovine spermatozoa under anaerobic conditions and converted in measurable amounts to radioactive carbon dioxide. An alternate possibility is that fructose might accelerate the entry of glycerol into the cell, as has been postulated by White *et al.* (27) for arabinose, a nonglycolyzable pentose.

After 105 min. of incubation, motility and per cent alive determinations were made on each flask shown in Figure 1. The data taken from this study

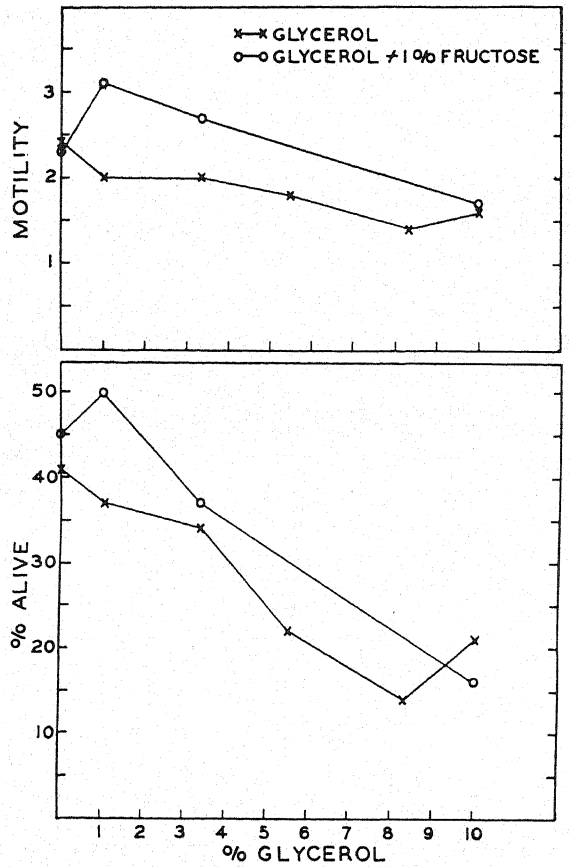


FIG. 2. Effect of various concentrations of glycerol and fructose (± glycerol) on spermatozoan motility and survival after 105 min. incubation at 38° C.

are presented in Figure 2. Mixner and Saroff (18) reported that glycerol levels above 4% in diluters containing egg yolk, skimmilk, or whole milk interfered markedly with the dead-alive stain; however, that effect was not noted in preliminary trials for these studies, where the spermatozoa were suspended in KRP salt solutions.

The results of the motility and per cent alive studies show that in flasks containing glycerol without fructose, motility and per cent alive were lower than in the corresponding glycerol-fructose flasks. The only exception was seen in the per cent alive studies with 10% glycerol. The most desirable motility rating was obtained with the samples containing 1% glycerol plus 1% fructose. Since more gas was produced in the 10% glycerol-fructose flasks, motility may have been depressed, due to a build-up of metabolites toxic to the cells instead of a direct toxic effect produced by the high concentration of glycerol. However, all flasks containing glycerol alone showed a lower motility, indicating a depression which probably was not associated with a build-up of toxic substances, since less metabolic activity, as measured by gas production, was observed in

these flasks than in the controls which exhibited a higher motility. This difference in motility between spermatozoa suspended in glycerol and glycerol-fructose might, in part, be explained by the report of Kampschmidt *et al.* (9), which suggests that as glucose is used to replace the buffer solution, viability of bovine spermatozoa is increased. This effect was attributed to: 1) more glycolyzable sugar present, or 2) decreased electrolyte content of the storage medium. Further support for the electrolyte view has been presented by Hendrikse *et al.* (7). They found that glycerol exerted a considerable apparent osmotic effect, with a lowering of the freezing point, when freezing bovine spermatozoa for use in artificial insemination.

One per cent glycerol in the presence of 1% fructose appeared to be more beneficial to the sperm cells, both from a motility and per cent alive standpoint, than any other combination studied. Since a rather large amount of metabolic activity was observed in these flasks without the decrease in per cent alive and motility, as observed in the 10% glycerol-fructose flasks, perhaps low concentrations of glycerol, in the presence of fructose, can be converted largely to carbon dioxide without the accompanying increase in toxic metabolic substances. Thus, a beneficial rather than a depressing effect was exerted on the motility and viability of the cells.

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TECHNICAL NOTES

ISOLATION OF IMMUNE GLOBULINS FROM MILK AND COLOSTRUM WITH RIVANOL[1]

Rivanol (2-ethoxy-6,9-diamino-acridine lactate) has been shown to precipitate quantitatively from blood serum the albumin and most of the α- and β-globulins (1, 2, 4). When applied to plasma, rivanol precipitates fibrinogen as well as albumin and α- and β-globulins. The γ-globulins remain in solution (1, 2, 4). A simple technique is thus afforded for isolation and analysis of the γ-globulins. It seemed worth while to determine whether rivanol can be employed in a similar fashion to fractionate the proteins of milk.

The technique described for blood serum or plasma was applied to separated colostrum, to acid whey from skimmilk, and to a crude lactoglobulin that had been isolated from acid whey by precipitation with 43% saturated ammonium sulfate. Specifically, the method consisted of adding 3.5 volumes of 0.4% aqueous rivanol[2] solution to one volume of solution to be fractionated, adjusting the pH to 8.5, centrifuging or filtering off the precipitated protein, and removing rivanol from the supernatant by adsorption on activated charcoal (2). The protein in the supernatant was analyzed electrophoretically in the Klett apparatus, after suitable concentration by pervaporation and dialysis against veronal buffer of pH 8.6, ionic strength = 0.1.

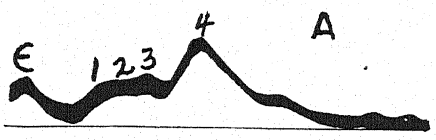
Figure 1 shows the electrophoretic patterns of crude lactoglobulin and of the filtrate obtained after treatment with rivanol. The filtrate contained primarily immune globulins, the faster components having been precipitated.

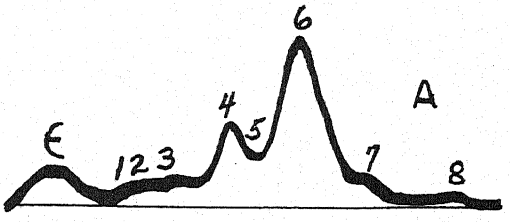
The term "immune globulins" is used herein to designate those protein fractions with mobility of -1.8 to -2.2 cm.^{2} volt^{-1} sec.$^{-1}$ at pH 8.6, ionic strength = 0.1 (4) without regard to the specific immunological properties of the components of such fractions. The components in all figures are designated numerically according to the usage of Larson and Kendall (5). Numbers 1 and 2 represent immune globulins, 4 α-lactalbumin, 6 β-lactoglobulin, 7 "blood" serum albumin, and 3, 5, and 8 the "proteose-peptone" constituents.

Figure 2 shows electrophoretic patterns of acid whey before and after treatment with rivanol. Again, the protein that was not precipitated consisted primarily of the immune globulins. The distribution of areas in Pattern B indicates that the material obtained from acid whey was 89.3% immune globulins. Furthermore, it was calculated from the electrophoretic analysis and nitrogen content of

[1] Paper No. 4071, Scientific Journal Series, Minnesota Agricultural Experiment Station. These studies were made possible in part by a grant from the Animal Disease and Parasite Research Division, Agricultural Research Service, USDA.

[2] Obtained from Winthrop Chemical Company under the name Ethodin.





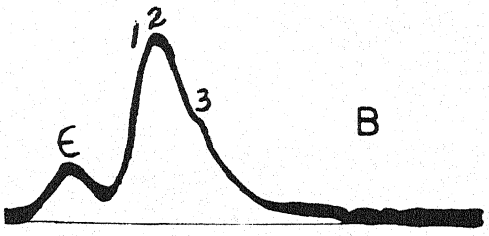


FIG. 1. Electrophoretic patterns of (A) "lactoglobulin" obtained from acid whey by 43% saturation with $(NH_4)_2SO_4$ and (B) fraction of "lactoglobulin" not precipitated by rivanol. Protein concentrations (A) = about 0.7%, (B) = 1.1%, time = 10,800 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

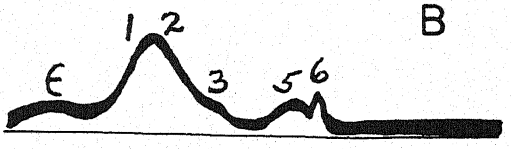


FIG. 2. Electrophoretic patterns of (A) whey proteins prepared from skimmilk by removal of casein at pH 4.6 and (B) fraction of whey proteins not precipitated by rivanol. Protein concentrations (A) = 1.5%, (B) = 1.2%, time = 10,800 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

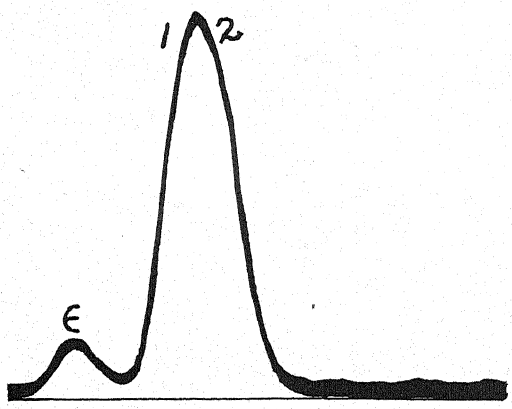


FIG. 3. Electrophoretic pattern of proteins of colostrum not precipitated by rivanol. Protein concentration = 2.8%, time = 13,540 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

the acid whey and its rivanol filtrate that 75% of the immune globulins of the acid whey were recovered in the rivanol filtrate.

Figure 3 is the pattern for a rivanol filtrate from colostrum.

The rivanol procedure is much more simple and rapid than the classical fractionation with ammonium sulfate, as a means of isolating the immune globulins of milk. Presumably, the globulins obtained in this way are not denatured (2), although this point needs to be checked more thoroughly. Recovery of the immune globulins would need to be improved and checked before the method could be used as a basis for quantitative determination of the content of immune globulins in milk.

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INFLUENCE OF VACUUM TREATMENT UPON MILK LOSS

The advent in recent years of nonsteam-injection vacuum equipment for the removal of off-flavors from fluid dairy products has created the need for research regarding its operation. The equipment utilizes the principle of boiling off volatile components in milk by the use of vacuum. Water vapors removed in the process result in concentration or loss of milk. This note deals with milk losses resulting from processing milk in various nonsteam-injection vacuum systems at various degrees of treatment or flash-cooling.

Mixed raw milk, thoroughly agitated in a storage tank and balance tank, was pasteurized at 172° F. for 16 sec. by a high-temperature, short-time pasteurizer. The milk was homogenized at 2,500 p.s.i. after pasteurization and vacuum treatment. Vacuum treatment was accomplished using various modified vacuum installations.[1] The condenser, when used, was a 2-in.-diameter stainless steel tubular type 4 ft. long, with city water circulating spirally in the outer water jacket. The condenser was installed at a rise of approximately a 30°-angle in the vacuum-vapor line as it left the top of the vacuum chamber. In the case of the double chamber, the condenser was similarly installed, but only over the first vacuum chamber. The flow of water through the condenser was adjusted to give a 3° F. drop in temperature of the vapors passing through the condenser.

[1] Vacu-Therm made by the DeLaval Separator Company, Poughkeepsie, New York.

Four replicates with four different degrees of flash-cooling increments were run on each installation. Duplicate total solids determinations by the Mojonnier method were made on the treated milk and the original raw milk samples. The difference in total solids between the raw and treated samples divided by the total solids of the raw sample, multiplied by 100, resulted in the calculated percentage milk loss. The method of least squares was used for determining the linear relationship between the degree of treatment and percentage milk loss. In cases where the relationship was curvilinear, the square root of the percentage of milk loss was used to make the relationship nearly linear.

The standard error of estimate was used as a measure of accuracy of the line (1).

The upward trend in the percentage of milk loss, with increasing degrees of flash-cooling in the various vacuum chambers, was expressed by the following equations, where Y represents the percentage of milk loss, Y_1 the square root of the percentage of milk loss, and X the degrees of flash-cooling: (1) $Y = -0.270 + 0.109\ X$ (single vacuum chamber before final heating); (2) $Y_1 = 0.004 + 0.061\ X$ (single vacuum chamber before final heating and with a condenser); (3) $Y_1 = 0.172 + 0.047\ X$ (single vacuum chamber after the flow-diversion valve and with a condenser); (4) $Y_1 = -0.052 + 0.035\ X$ (double vacuum chamber with the first chamber before final heating and the second chamber after the flow-diversion valve); and (5) $Y_1 = -0.084 + 0.034\ X$ (double vacuum chamber as noted in four, plus a condenser). The standard error of estimate for the first equation was 0.19% milk loss, while for the remaining equations it was, respectively, 0.12, 0.15, 0.11, and 0.09 for the square root of the percentage of milk loss.

The equations for estimating the per cent milk loss from degrees of flash-cooling, in instances where a condenser is not used, are valid for similar nonsteam-injection vacuum equipment, with minor changes for radiation-loss variances. In cases where a condenser is used during treatment, the equations are not valid unless identical conditions exist, but they indicate the approximate expected milk loss.

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RECOVERY OF ADDED MONOGLYCERIDES FROM MILK

Monobutyrin is water-soluble and is probably the monoglyceride of shortest chain length that would be found in lipolyzed milk. In a previous paper (2), some of the difficulties encountered in recovering added monobutyrin from milk were described. Commercial monobutyrin contains glycerol which analyzes as if it were two moles of monoglyceride. The poor recoveries of monobutyrin encountered from milk were due to the retention of glycerol in the silica-gel when this method was used to extract monoglycerides. Thus, the difficulties centered about the purification of monobutyrin. This has been accomplished with the method of Gander (1), in which monobutyrin is eluted from a celite-silicic acid column that retains the

TABLE 1

Extraction of added monostearin and monobutyrin from milk by the silica-gel method

	Monostearin[a]		Monobutyrin[a]	
	Added	Found	Added	Found
	(mg. per 100 ml. milk)		*(mg. per 100 ml. milk)*	
2.0-m*M* level	23.00	22.30	14.94	14.10
	24.80	23.90	13.65	13.59
	23.00	22.20	13.00	12.59
	24.68	24.72	11.80	11.52
			15.77	14.98
Mean	23.87	23.28	13.83	13.36
Standard deviation of a sample[b]		0.64		0.31
Per cent recovery		97.5		96.6
0.1-m*M* level	1.27	1.27	0.75	0.69
	1.14	1.16	0.65	0.62
	1.31	1.31	0.65	0.59
	1.21	1.23	0.59	0.62
			0.79	0.86
Mean	1.23	1.24	0.69	0.66
Standard deviation of a sample		0.037		0.029
Per cent recovery		100.8		95.7

[a] Corrected for purity.
[b] Standard deviation calculated from the difference between duplicates.

glycerol. This paper describes the recovery of purified monobutyrin and monostearin from milk with the silica-gel extraction method.

Monostearin was purified by washing an ether solution with water to remove glycerol, and had a total monoglyceride content of 83.8%. Monobutyrin was purified as described above, and had a total monoglyceride content of 92.2%. Both were added to pasteurized milk at levels of 0.1 and 2.0 m*M* per 100 g. of fat. The monostearin was dispersed with a hand-operated homogenizer at 70° C. The extractions from milk and analyses of monoglycerides were performed as described in the previous paper (2). At the 2 m*M* level, 25 ml. of milk were extracted, the solvent evaporated, and the fat rinsed into a 25-ml. volumetric flask. At the 0.1 m*M* level, 50 ml. of milk were extracted and the extracted fat rinsed into a 10-ml. volumetric flask. The results are presented in Table 1. The data in Table 1 show that monoglycerides added to milk are extracted quantitatively by the silica-gel method.

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EFFECT OF SOME PRESERVATIVES ON BABCOCK TESTS AND FAT HYDROLYSIS IN SINGLE MILK SAMPLES[1]

Manus and Bendixen (4) showed that the addition of corrosive sublimate reduced the Babcock test reading and caused large increases in the free fatty acid titers of composite milk samples.

The object of this study was to compare the effect of several common preservatives on the Babcock test reading and the free fatty acid titer of milk samples stored for 1 and 2 wk.

EXPERIMENTAL PROCEDURE

In the first series of tests, seven lots of mixed Grade A raw milk from the College dairy were each split into five portions and treated as follows: (a) no additions; (b) addition of two No. 2 corrosive sublimate ($HgCl_2$) preservative tablets (0.45 g. each) per 440 ml. of milk; (c) addition of three Milkeep[2] preservative tablets (0.325 g. each) per 420 ml. of milk; (d) addition of four potassium dichromate preservative tablets per 400 ml. of milk, and (e) addition of 1 ml. Mojonnier test fluid per 455 ml. of milk.

In the second series, ten lots of mixed Grade A raw milk were each split into five portions and treated as follows: (a) 0.5 g. CP potassium dichromate per 400 ml. of milk; (b) 0.5 g. CP potassium chromate per 400 ml. of milk; (c) three tablets Milkeep per 420 ml. of milk; (d) 1 ml. Mojonnier test fluid per 455 ml. of milk, and (e) 0.6 g. CP mercuric chloride per 440 ml. of milk.

To dissolve the preservatives rapidly, all tablets were pulverized prior to addition to the milk.

Immediately after all additives were completely dissolved, milk for six Babcock test bottles was pipetted from each sample, to avoid possible errors due to fat adhering to the bottle surface after storage. Care was taken also to prevent any churning during the mixing of the sample before pipetting. Within 60 min., Babcock tests (1) were completed on the samples in two of the test bottles. They were repeated in duplicate after storage at 35–38° F. for seven and 14 days.

Triplicate samples from each preserved portion were placed in half-pint milk bottles. Immediately after the Babcock tests were completed, the free fatty acid determinations were made in duplicate, using silica gel chromatography as described by Harper *et al.* (3), except that 2 ml. of 2% phenolphthalein in absolute ethanol was used as the indicator. The free fatty acid titer tests were repeated in duplicate after storing the samples at 35–38° F. for seven and 14 days.

Differences between the average of the fat percentages obtained immediately after the additives were in solution, and the average of the percentages obtained after storage at 35–38° F. for seven and 14 days, were recorded as the observed reductions in the fat percentage during storage. The increase in free fatty acid titer was obtained in a similar manner.

The Babcock test bottles were selected for an accuracy of ± 0.05%, or less.

[1] Scientific Paper No. 1835, Washington Agricultural Experiment Stations, Pullman. Project 839.

[2] Active ingredients according to the manufacturer's label: Disodium phosphate, 3, 5-dimethyltetrahydro 1, 3, 5, – 2H–thiadiazine-2-thione.

RESULTS AND DISCUSSION

The results obtained in comparing nonpreserved and corrosive sublimate–preserved samples were essentially the same as previously reported by Manus and Bendixen (4). It did not matter whether commercial preservatives were used as in Series 1 or whether the chemically pure preservatives were used as in Series 2. Therefore, data for the second series only will be presented.

In Figure 1, the average fat percentage reduction during storage is shown to be small for the samples preserved with chromates, Milkeep, or Mojonnier test fluid, whether the holding period was 1 or 2 wk. However, mercuric chloride reduced the average Babcock percentage more than three times as much as the chromate-preserved samples, and almost doubled that for the samples preserved with Milkeep or Mojonnier test fluid.

Duncan's (2) multiple range and multiple F test shows the reduction in Babcock fat percentages to be significantly greater at the 1% level when mercuric chloride was used as a preservative than when any of the other four preservatives were used. There were no statistically significant differences in the reduction in fat percentages between the samples preserved with potassium chromate, potassium dichromate, Milkeep, or Mojonnier test fluid at the 1% level.

The increase in free fatty acids with the different preservatives as shown in Figure 1 was not statistically analyzed. However, the data show that free fatty acid increases were generally accompanied by a reduction in the Babcock fat test. The increase in free fatty acids and the reduction in the Babcock fat test were considerably greater for the samples preserved with mercuric chloride than for those preserved with the other preservatives.

Figure 2 compares both the fat percentage reduction and the free fatty acid titer increase for 1 and 2 wk. in single samples, when potassium dichromate and mercuric chloride are used as the preservatives. Generally, a larger reduction in the butterfat test is accompanied by a

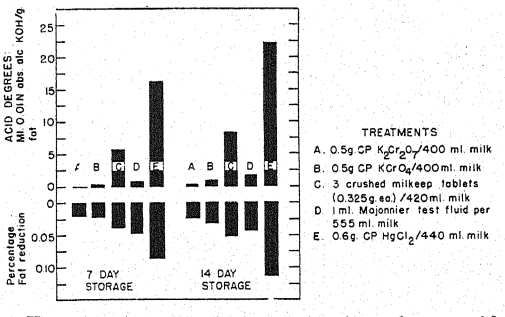


FIG. 1. Average increase in free fatty acids compared with average Babcock fat test reduction in ten single samples of preserved milk stored at 35 to 38° F. for seven and 14 days. (All tests in duplicate.)

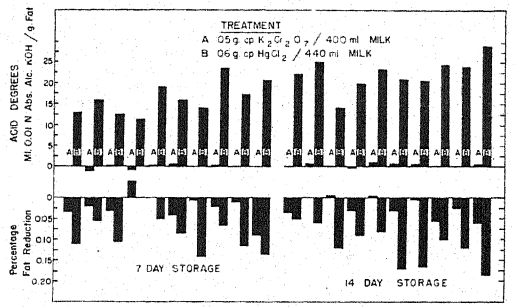


FIG. 2. Increase in free fatty acids compared with Babcock fat test reduction in ten single samples of preserved milk stored at 35 to 38° F. for seven and 14 days.

larger free fatty acid titer. The correlation was fair.

The reason why greater fat reductions and a large increase in free fatty acids occur with mercuric chloride is not clear.

Mercury is a protein precipitant and one may, therefore, speculate that the protein-lipid complex membrane surrounding the fat globules is precipitated by the added mercuric chloride, thus increasing the exposure of the fat to lipase activity. The mercury ion is a well known inhibitor of some enzymes. However, it appears to improve the conditions for lipolysis. Raw milk samples preserved with mercuric chloride, stored at 35–50° F. for a week or longer, invariably have a definitely rancid odor. No odor was detected in samples preserved with the chromates. The odor of samples preserved with Milkeep was that of wet phosphorus matches. Any odor that might have developed in the samples preserved with Mojonnier test fluid was masked by the formaldehyde in the test fluid.

SUMMARY

Results reported previously showing an acceleration in fat hydrolysis and a significant decrease in the fat test of milk samples preserved with mercuric chloride have been corroborated.

Milk preservatives, such as potassium dichromate, potassium chromate, Milkeep, and Mojonnier test fluid yielded results which are not significantly different at the 1% level. Mercuric chloride caused a significant lowering at the 1% level of the fat test and markedly increased the free fatty acid titer in single milk samples stored at 35–38° F. for 1 and 2 wk.

ACKNOWLEDGMENTS

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EFFECT OF FEEDING COD LIVER OIL AND UNSATURATED FATTY ACIDS ON RUMEN VOLATILE FATTY ACIDS AND MILK FAT CONTENT

The depressing effect on the fat content of milk induced by the feeding of cod liver oil to cows has been known since the early 1920's [see review by Hilditch (2)]. Hilditch has proposed that the unsaturated fatty acids of the C_{20} and C_{22} series are responsible for this depression, possibly by exerting a poisoning effect on mammary enzyme systems. Hydrogenation of the cod liver oil eliminates the effect.

In view of high correlations observed between the molar per cent of rumen acetic acid and fat content of milk, and an equally high negative correlation between the molar per cent of rumen propionic acid and fat content of milk (9), it appeared that cod liver oil might exert its effect on fat content of milk by altering the proportions of the rumen volatile fatty acids (VFA). Accordingly, the rumen VFA of six cows were determined before and after the initiation of the per os administration of 150 ml. each morning and evening of cod liver oil, oleic acid, or linoleic acid.

All cows had been on constant feeding regimens for over a month prior to the administration of these additives. Three of the cows had been on a normal diet and three had been on diets designed to decrease the fat content of milk (1, 7). An additional cow receiving a normal diet of alfalfa hay and a mixed concentrate was fed 150 ml. of oleic acid twice per day, to ascertain the effect on the composition of milk fat. The following techniques were used: rumen VFA, a modification of the method of Wiseman and Irvin (10); iodine number, Rosenmund and Kuhnhenn (6); lower fatty acids of milk fat, a modification by Lakshmanan and Mougey in this laboratory of the Keeney method for butyric acid (3). Rumen samples were obtained 3 hr. after feeding by means of a large rumen tube (Colorado tube); the supernatant fluid obtained following centrifugation of the rumen contents was frozen for purposes of preservation and analyzed within three days.

The data presented in Table 1 show that cod liver oil, oleic acid, and linoleic acid, when administered to cows on normal diets, decreased fat content of milk and exerted a remarkable effect in (1) decreasing the molar proportion of rumen acetic acid and increasing the molar proportion of propionic acid, and (2) increasing the concentration of total VFA in the rumen fluid, linoleic acid having the greatest effect.

When given to cows on diets which had already decreased milk fat content markedly, linoleic acid once again had the greatest effect (Cow 175) on the rumen VFA, being complementary to the basal diet in decreasing the acetate and increasing the propionate proportions and in increasing the concentration of total rumen VFA. Cod liver oil also had a complementary effect (Cow 242), but to a lesser degree in effecting these changes. Oleic acid was complementary to the basal diet (Cow 164) in increasing the concentration of total VFA in the rumen fluid, but did not effect a further change in the molar proportions of rumen acetate and propionate. However, it will be noted in this case that the basal diet had already effected very large changes, the molar proportion of propionate being greater than that of acetate. The effect of feeding oleic acid on the short-chain fatty acids of milk fat is shown (Table 2). There was a decrease in the molar percentage of each of the lower fatty acids, an increase in iodine number, and a decrease in fat content of milk (25%) similar to that produced by feeding cod liver oil (2).

The effect of the additives on the fat content of milk of cows already on low-milk fat producing diets suggests some interesting possibilities. The failure to get a further depression in fat content of milk from the feeding of oleic acid (Cow 164) or linoleic acid (Cow 175) probably was due to the low levels attained on the previous diets. In Cow 242, which was fed cod liver oil, there was a slight increase followed by a decrease in the fat content of milk. The tendency of the milk fat content either to increase or undergo only slight decreases in Cows 242 and 164, in which the previous diets had effected decreases in the fat content of milk, could be explained on the basis of the earlier postulation of Shaw and Lakshmanan (8), in which it was proposed that a decreased synthesis of short-chain fatty acids (mammary lipogenesis) was ordinarily compensated to some extent by an increased uptake of longer-chain fatty acids from the blood, and vice versa. In the present instance, the fatty acids other than the C_{20} and C_{22} acids of cod liver oil may

TABLE 1

Effect of feeding cod liver oil (CLO), oleic acid (OA), and linoleic acid (LA) upon rumen volatile fatty acids (VFA) and fat content of milk

Cow	Basal diet	Additive	Time after feeding additive	Milk fat	Molar % of total rumen VFA					Total rumen fluid VFA
					C_2	C_3	C_4	C_5	C_6	
	(lb/day)	*(ml/day)*	*(hr.)*	*(%)*						*(mg/100 ml.)*
	Alfalfa hay (AH) + mixed concentrates (MC) + additives									
SK-1	22 AH + 11 MC	300 Cod liver oil	0	2.9	71.1	14.1	12.5	1.9	0.4	635.9
			63	2.4	56.1	28.2	9.1	5.2	1.4	787.8
S-1 1	12 AH + 10 MC	300 Oleic Acid	0	5.0	67.4	15.4	11.8	5.3		448.0
			63	4.5	61.4	21.5	14.3	2.7		870.1
K-1	12 AH + 12 MC	300 Linoleic Acid	0	4.0	78.9	9.9	7.7	3.4		584.2
			63	2.6	52.2	29.9	14.0	3.8		762.4
	Ground and pelleted alfalfa hay (GPAH) + heated corn (HC) or ground unheated corn (GC) + additives [a]									
242	30 GPAH + 4 GC	300 Cod liver oil	0	2.7	65.9	22.9	8.5	2.0	0.8	1,357.6
			24	2.9	62.7	26.1	7.8	2.4	0.9	1,426.2
			48	2.7	60.1	28.3	8.5	2.5	0.5	1,540.1
			72	2.5	59.6	28.4	8.5	2.8	0.8	1,549.1
164	6 GPAH + 16 HC (steam 275° F.)	300 Oleic Acid	0	1.7	43.7	47.3	3.7	4.2	1.1	948.7
			63	2.3	48.2	45.5	4.3	1.8	0.2	1,162.5
175	6 GPAH + 16 HC (Dry heat 400° F.)	300 Linoleic Acid	0	1.4	54.9	32.0	7.9	4.4	0.9	677.3
			63	1.5	46.1	41.7	7.7	3.8	0.6	1,579.1

[a] Extent of depression of fat content of milk by basal diets prior to feeding of additive: Cow 242, 25%; Cow 164, 47%; Cow 175, 59%.

TABLE 2

Effect of feeding oleic acid to a cow on milk fat composition

	Iodine number	Fatty acids of milk fat in molar %				
		C_4	C_6	C_8	$C_{10} + C_{12}$	Higher
Prior to feeding oleic acid	31.4	8.9	5.2	2.9	6.6	76.4
After feeding oleic acid three days (300 ml. per day)	49.5	4.8	2.6	2.1	4.3	86.2

have compensated to some extent for the decreased mammary lipogenesis. Oleic acid could have both an increasing and a decreasing effect, the former prevailing when mammary lipogenesis was already inhibited markedly, as in the case of Cow 164. Since linoleic acid is not normally taken up by the mammary gland in appreciable amounts, it would not be expected to have a compensatory (increasing) effect on fat content of milk.

We have noted (unpublished) that the basal milk fat depressing diets used here decrease the lower fatty acids of milk fat. These considerations would explain the variable results on fat content of milk obtained from the feeding of oils other than cod liver oil [see Petersen (5), and Moore *et al.* (4) for review]. The observations of Moore *et al.*, that fat content of milk was not decreased when cod liver oil was administered per os in small amounts but at frequent intervals, can be understood on the basis that the concentration of the C_{20} and C_{22} unsaturated fatty acids in the rumen was insufficient to produce the necessary changes in the rumen VFA.

It is conceived that the above findings not only explain the long-known effect of cod liver oil on the fat content of milk, but also offer considerable potential for influencing animal performance by controlling the proportional (and total) production of rumen VFA by means additional to those previously demonstrated (1, 7). Oils containing long-chain, highly unsaturated acids, unsaturated fatty acids per se and, possibly, certain unsaturated hydrocarbons when added to rations for ruminants, would be expected to produce the above-noted results in greater or lesser degree, depending upon the amount and degree of unsaturation of the additive. Theoretically, this should apply to pasture as well as to dry-lot feeding.

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OUR INDUSTRY TODAY

PRODUCTION OF QUALITY PROCESS STEAM

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Explanation of terms used, and review. In this presentation, the terms direct heating and indirect heating will be used rather frequently. The former refers to the direct mixing of steam with the product processed. The latter, of course, refers to steam used for heating a product through a heat-transferring surface.

If steam were used indirectly for processing dairy and food products, the only possible contamination of such foods by steam contaminants, aside from leaks, could be by residues adhering to product surfaces as a result of cleaning solutions prepared by mixing steam and water, or from sterilization of equipment by use of direct heating with steam.

Currently, steam is used directly in the dairy industry for foam heating in batch pasteurization, as a requirement by most city milk ordinances. It is also used in some vacuum-type pasteurizers for milk and cream, more recently in vacuum treatment of milk and cream to remove foreign volatile flavor and odor compounds, in the preparation of processed cheese or cheese foods, and in the preparation of some concentrated milk products, such as superheated skimmilk etc. Earlier in the dairy industry, direct steam heating was traditionally practiced for preheating milk in hot wells before concentrating.

With the advent of improved controlling equipment, and in line with newer knowledge of milk constituents, there is a tendency on the part of certain branches of the industry to favor higher processing temperatures, with correspondingly shorter holding periods.

For some time, research has been in progress to establish a time and temperature relationship when heating is carried to much higher levels than those currently suggested in the United States Public Health Service Standard Milk Ordinance and Code.

One of the principal reasons for the somewhat slow progress in this direction has been the absence of sufficiently sensitive controlling equipment. Progress is being made in this direction.

The term ultra-high heat processing has been applied to temperature treatments which are considerably in excess of the present standard for High-Temperature–Short-Time (HTST) pasteurization. Temperatures currently under consideration approach or even exceed 300° F. The time of exposure to temperatures of this magnitude would be in the nature of a fraction of a second. Even with a heavy inoculation of spore formers, sterile milk has been produced at 297° F. with a 0.5-sec. holding time.

Certain problems with respect to heating equipment may be anticipated. Nevertheless, several manufacturers have equipment of this kind on the market. It is recognized that the problem could be greatly simplified if direct heating by the introduction of steam to the product were permissible. Sanitarians will have to be convinced that this type of heating is safe from the standpoint of the ultimate consumer.

Currently in this country, ultra-high heating is accomplished by use of indirect high-velocity tubular type or by swept-surface heat exchangers. Each of these minimize "burn-on," a problem with all indirect high-temperature milk heaters. Many feel that direct heating is the best answer.

The food-processing industry is using direct steam heating for various treatments. It is used for blanching and for peeling certain vegetables, for humidity control in bakery fermentation chambers, in the preparation of some fruit products such as peeling peaches, mixing with apple sauce, in the brewing industry for heating mash, etc.

Direct mixing of steam and water for cleaning and rinsing solutions is regaining some of its former popularity as a result of the development of greatly improved mixing chambers.

There is one big difference between processing dairy products and most other foods. Because of the unusual position of dairy products in the diet and their use in liquid form by infants, children, expectant mothers, and by invalids, where it sometimes constitutes the entire diet, a special problem of protection from contamination is created. Cheese or cheese foods, on the other hand, apparently are frequently classified along with fruits and vegetables with respect to tolerances for food additives.

In a number of industries where large quantities of steam are used, there has been a trend toward high-pressure generators. This necessitates specialized water-conditioning methods. New chemicals for inhibiting corrosion have been developed and, because of their effectiveness, they are also often included in water-conditioning compounds to be used in boilers operating under 200-p.s.i.g. pressure.

Water and steam conditioners as food additives. Regulatory agencies, processors, and a number of consumer groups agree that if residues from boiler water conditioners enter dairy and food products by way of direct heating, they should be regarded as additives. The Federal Food and Drug Administration undoubtedly will apply Sec. 402 (a) (1) of the Federal Food, Drug, and Cosmetic Act, which

reads in part: "A food shall be deemed to be adulterated . . . if it bears or contains any poisonous or deleterious substance which may render it injurious to health . . .;" and/or it may apply Sec. 406 (a) which reads in part ". . . except where such substance is required in the production thereof or cannot be avoided by good manufacturing practice, shall be deemed to be unsafe . . . but when such substance is so required or cannot be so avoided, the Secretary shall promulgate regulations limiting the quantity therein. . . ." Presumably, this gives the Federal Food and Drug Administration considerable latitude of interpretation. We do know that in the case of DDT, tolerances are zero for dairy products, but tolerances for fruits and vegetables may range from 1 to 5 p.p.m., depending upon the quantities of such food products present in usual diets.

The new food additive amendment to the Federal Food and Drug Act places the burden of proof of nontoxicity of a food additive on the manufacturer. I do not want to discuss the merits of this legislation but, if you are planning to use steam for direct processing, it is well to know what ingredients are present in feed-water conditioning compounds before proceeding with their use. As a result of the new amendment, we may expect new water-conditioning materials to reach consumer channels at a faster rate than was the case formerly. The manufacturer now has the alternatives of testing the product in his own laboratories or having it tested by a reputable private testing laboratory, or through the medium of an industrial grant to a recognized university or college, which will undertake to carry out the necessary tests and publish the findings.

Another factor which processors of foods should not disregard is the greatly improved techniques which have been developed or are in the process of development for detecting even minute quantities of specific additives which may be present in a food product.

Advantages and disadvantages of direct heating. A processor of dairy or food products would not be inclined to use direct steam heating unless the product can not be handled otherwise, or where it has distinct advantages. In the case of dairy products, the advantages of direct steam heating may be summarized somewhat as follows:

1) Lower processing costs, because
   (a) Smaller and less costly processing equipment may be used. On a theoretical basis, the area of a processing unit of a direct heating machine would have to be only one-eightieth of that of an indirect heater of comparable capacity.
   (b) Direct heating results in increased efficiency. This is especially true of products with a high Reynolds number (high viscosity) and turbulent flow.
   (c) Labor costs are less because cleaning operations are simplified.
2) Danger from biological recontamination is reduced.
3) Off-flavors due to high temperatures on heat-transferring areas of indirect heating equipment are reduced when direct heating is practiced.
   (a) Care should be exercised so as to avoid the use of superheated steam in direct heating, since this may result in protein denaturation and subsequent flavor defects.
4) Lower boiler pressures are possible.
5) Lower radiation losses; hence, better operating efficiencies result.

Objections to direct heating with steam include:

1) Danger of carry-over of conditioning compounds due to faulty boiler operation, such as
   (a) Overcrowding a boiler.
   (b) Foaming and priming.
2) Possibility of presence of toxic volatile corrosion inhibitors in the steam.
3) Increased amounts of make-up feed water will result in higher conditioning costs.
4) Increased scale formation will occur unless special care is exercised in conditioning the feed water.
5) More frequent blow-down or deconcentration will be necessary, thereby decreasing the efficiency of steam generation.
6) Flavor defects resulting from the presence of conditioning compounds may occur.
7) Phosphate positive reactions are more apt to result where direct heating is practiced even at the higher tempraturs, e.g., 200° F. for 0.7 sec.

An examination of the advantages and disadvantages indicate that those concerning some aspects of efficiency might cancel out. This is true of greater heating efficiency versus increased water-conditioning costs. For the time being, at least, these items will not be considered.

The immediate problem is to convince regulatory agencies and consumer groups that direct heating with steam is at least as satisfactory as, or superior to, indirect heating.

Equipment which will pass present sanitary standards for processing dairy products by direct steam heating is presently available. In the past, the problem of adequate controlling equipment for ultra-high heating limited its development. This is being rapidly overcome. Nevertheless, we must learn more about lag phases in the temperature of the various constituents in the product processed.

Steam need not necessarily contain toxic compounds in order to be objectionable. It may cause flavor or color defects, which do not con-

cern regulatory agencies. On the other hand, if it causes dilution, when such dilutions are not permissible, it may involve legal interpretations. If the product is diluted, what assurance has management that prescribed practices are followed during processing to counteract the dilution?

The plant engineer is most concerned with scaling and corrosion in the operation of a steam-generating plant. Boiler water conditioning practices are developed to overcome these problems. Most specialized treatments are generally directed toward the elimination of corrosion. High-pressure steam will usually cause more rapid corrosion than low-pressure steam. High-pressure boilers are most often encountered in steam electric generating plants, chemical plants, and industrial enterprises, rather than in dairy or food plants. For this reason, water-conditioning engineers have given much greater attention to these enterprises than to dairy and food plants.

Dissolved oxygen and/or carbon dioxide, plus improper pH control, are responsible for most corrosion problems. Some of the newer chemicals used as components of boiler feed water conditioners were developed for operations where contamination of foods was not a problem. However, when an engineer in a dairy or food plant encounters corrosion difficulties, he consults boiler conditioner manufacturers, who may not appreciate the problems of the food plant or who are not aware that steam is to be used for direct heating.

Boiler water and steam conditioners. Various techniques have been developed for overcoming the problems of scale formation on boiler surfaces or of corrosion within the boiler or in condensate return lines and processing equipment.

Conditioning of water prior to its entry to the boiler includes settling tanks, sand filters with or without coagulants, heat treatment, the use of calcium hydrate, sodium carbonate, various aluminates, and/or by the use of ion exchange equipment of various types. Electronic devices also have been advocated. For the elimination of dissolved oxygen, beds of steel shavings or sulfite treatments have been used. Reboilers also have been suggested.

For conditioning water inside of boilers a wide variety of materials is available from manufacturers of boiler compounds. The chief functions of such compounds are the replacement of objectionable water impurities by the formation of less objectionable compounds, by precipitation, or by formation of colloidal suspensions, and in some cases by film formation on susceptible surfaces as well as neutralization of oxygen and carbon dioxide activity.

Boiler water conditioning compounds (internal treatment) include sodium hydroxide, sodium carbonate, sodium sulfite, sodium polyphosphate, sodium orthophosphate, heavy phosphates, inhibited sulfanic acid, sodium alginates, quebracho,[1] phenolic type compounds such as tannins and lignins, starch, agar, gelatine, glycerine, petroleum derivatives, hydrazines, chromates, volatile or filming amines, and even sugar, whole potatoes, and cheese whey.

Contamination of steam by impurities in water or by conditioning compounds can occur by volatilization or by simple carry-over (entrainment). Volatile compounds in the form of neutralizing agents may be deliberately added to the boiler water, to protect condensate lines from corrosion. They serve a useful purpose where heating is by indirect means and the condensate is brought back to the boiler. If heating is by direct steam and no condensate is brought back to the boiler, there is less justification for the use of corrosion-inhibiting compounds. Tannins and lignins were regarded with suspicion by regulatory agencies for some time, but recently their attention has been focused on a group of compounds which includes such nonwettable film-forming long-chain amines as octadecylamine and octadecylamine acetate, and on such neutralizing amines as cyclohexylamine and morpholine. For this group of compounds there is as yet no established tolerance in dairy products consumed in fluid form. That means zero tolerance. Federal Food and Drug Administration interpretations for tolerances for additives to steam used for direct heating of food products (specifically, milk consumed in fluid form) mainly are based on naval specifications.

There is some indication that some agencies are relaxing their requirements. The Bureau of Medicine and Surgery of the U.S. Navy indicated in a letter dated July 3, 1958, that on the basis of a report from the National Academy of Science, National Research Council, the addition of octadecylamine as an anticorrosion agent in steam for cooking foods is acceptable at levels no higher than 2 to 3 p.p.m., on the basis of need or emergency. No mention is made of milk consumed in fluid form.

A more recent communication (October 13, 1958), from the Commander of the U. S. Navy Power and Utilities Branch, Maintenance Division, contained this significant statement: "On the basis of recent test data, however, it appears that this bureau may soon be in a position to relax the present restriction concerning 'food contact' on the premise that injection of octadecylamine into the system (steam) can be so controlled that its concentration in the steam will not rise to levels higher than 2 to 3 parts per million."

If the present trend toward increased application of direct heating of dairy and food products continues, it is up to the industry to develop equipment and practices which will be acceptable to regulatory agencies and to the consuming public.

[1] South American tree, *Quebrachia lorentzii*, the bark of which has much tannin.

Handling steam without the use of toxic conditioners. A number of steps may be suggested. One is a suggestion that boilers, piping, and fittings be constructed of stainless steel. A poll of boiler manufacturers brought an almost unanimous negative reaction. First, the cost would be from eight to ten times (more for smaller boilers) higher than for carbon steel boilers. Furthermore, there appeared to be considerable doubt that the all-stainless boiler would outlast or outperform carbon steel boilers. It appears that cases of chloride stress corrosion and failure of stainless steel boilers have occurred after relatively short periods of operation. To entirely eliminate chlorides from boiler water is prohibitive in cost. Metals other than carbon steel which have been used successfully in boilers include both copper-nickel and nickel-copper alloys. These alloys have the added advantage of high-heat transference coefficients.

Corrosion of the boiler surfaces where properly conditioned water is used is slight and, hence, contamination of the steam by metals or metallic salts formed from the boiler plate is slight. There is, however, greater danger of metallic contamination from steam piping, especially if operation is on an intermittent basis. There might be some justification for the installation of stainless steel piping and fittings if direct steam heating is practiced. This would reduce the necessity for the use of corrosion-inhibiting compounds.

As a rule, the higher the pressure at which a boiler is operated, the more intense the activity of corrosion-promoting agencies. The dairy and food industry is not concerned with extremely high boiler pressures, such as 1,200 to 3,000 p.s.i.g. A pressure of 52 p.s.i.g. is sufficiently high to attain a temperature of 300°F. when direct heating is practiced, but a pressure of 100 p.s.i.g. or higher will be necessary for similar temperatures when heating is by indirect means. Neither of these pressures is considered high.

From the standpoint of solids content, steam at its worst (90% quality) would not carry in excess of 0.04% solids, but specialized steam separators or purifiers are available for installation within the boiler or steam drum, or between the boiler and processing equipment, to remove even such solids. Traps and strainers in the line are standard in most installations. These accessories can be designed to reduce the solids carry-over in steam to approximately 1.0–2.0 p.p.m., when the boiler is operated at less than 3,500 p.p.m. of solids in the water. With highly specialized separators the solids carry-over may be brought down to 0.3 p.p.m.

The safety of additives in boiler water or to steam depends upon management and the plant engineer. Boiler feed-water conditioners or steam additives should be purchased only after checking with management and the plant laboratory.

Management should consult with the plant engineer to assure a boiler of ample capacity, so that it need not be forced. The boiler should have the proper design for a possible fluctuating load. Boiler salesmen should not recommend a type or size of boiler which obviously is not suited to the purpose for which it is to be used.

In the installation of steam lines, lead or other toxic jointing compounds should be avoided. The boiler and the steam lines should be thoroughly cleaned before the plant is placed in operation for direct heating.

The blow-down of the boiler should be carefully watched, so that over-concentration and foaming are avoided. In many cases, a continuous blow-down system has an economical advantage. For dairy and food plants a solids concentration of 3,500 p.p.m. in the boiler should be considered as maximum.

Some dairy and food plant architects, for reasons of their own, have advocated desegregation of steam-generating equipment by placing automatic-type boilers adjacent to various processing areas. The same idea could be extended to the use of a special boiler for producing steam to be used for direct injection. This would permit a water-conditioning procedure to exclude toxic compounds of any kind.

Where steam for all purposes is produced in one common steam-generating plant, and if corrosion of condensate return lines is a problem, certain precautions may be observed. The substitution of starch for other corrosion inhibitors, such as tannins, lignins, chromates, and amines, may serve the purpose with somewhat less desirable results. Special phosphate-alkaline formulations have been developed for direct injection into steam lines for film production and acid neutralization. In the amounts necessary, they are nontoxic. Special alloys for condensate return lines might be advisable.

Since oxygen and carbon dioxide are mainly responsible for corrosion, special techniques might be used for removing these gases before the feed water enters the boiler. Some of these have been previously indicated. One concern which required steam of exceptional purity solved the problem of corrosion and scaling by installing a two-stage steam generator. A gas-fired heater is used to heat a refined heat-transfer oil to 500° F. The hot oil is passed through a tubular heat-transfer steam generator for converting 200° F. preheated water into steam. From the oil-heated steam generator, the oil which still carries heat passes through a double-jacketed water heater in which the cold feed water is preheated to 200° F. Scale will form on the surface of both heaters, so that construction must be such that they can be easily opened for descaling.

Sodium sulfite is frequently added to boiler feed water as an oxygen-reducing agent. The amount may range from 20 to 50 p.p.m. The concentration in the carry-over could reach 200 to 500 p.p.m. Sulfites are permitted as ad-

ditives in a number of foods and, hence, should not be regarded as too objectionable for use in boiler water treatments, except in the case of milk consumed in fluid form.

One aspect of the oxygen problem is that of prevention. Improperly sealed centrifugal condensate return pumps may add from 200 to 600 p.p.m. of oxygen by volume. Water itself may be a contributing factor. Oxygen solubility may reach 16,000 p.p.m. by volume, but probably averages closer to 6,000 p.p.m. For most boiler-operating conditions, it should be kept below 30 p.p.m. by volume in the boiler, although some operators insist that it may reach 100 p.p.m. without causing a serious problem.

Proper adjustment of the pH of boiler water is helpful in reducing corrosion. Iron is least soluble at pH 9.6, but this is unsatisfactory for other metals or alloys in a steam system and, hence, it is customary to maintain a pH of 8.6–9.0.

The discussion up to this stage has been based on the assumption that any toxic corrosion inhibitor must be kept completely away from the product, which is heated directly with steam. If steam were 100% in quality, none but volatile compounds would be carried over. Suppose, however, that it were 95% quality and that the solids concentration of the 5% carry-over were 3,500 p.p.m. If such steam were at 52 p.s.i.g. pressure, and if it were used to heat milk directly from 35 to 300° F. with no attempt to use a steam separator or traps, the amount of solids added to the milk-condensate mixture would amount to 32 p.p.m. If an amount of water equivalent to the condensate were then evaporated from the mixture, the solids added by the steam would be slightly under 40 p.p.m. in the product.

Toxicity of amine types of conditioners. Where filming amines such as octadecylamine are added, the usual dosage is at the rate of 1 to 3 p.p.m. of steam and, hence, the concentration in the milk condensate mixture would be less than 1 p.p.m.

Extensive studies have been done on the toxicity of octaldecylamine by Dr. W. B. Deichmann,[2] who is quoted as follows: "No toxic effects whatever, due to octadecylamine, were observed when this compound was fed in the diet to rats for two years at levels up to 500 p.p.m. The weight gains, blood counts, survival, and food consumption of the rats fed octadecylamine were indistinguishable from the controls. No significant pathological changes due to octadecylamine were observed when the tissues of these animals were examined microscopically."

[2] University of Miami, School of Medicine, Coral Gables, Florida.

Dogs likewise were used in the experiments, and the final statement follows: "Thus it would appear from this investigation that the maximum nontoxic levels of octadecylamine are 500 p.p.m. for the rat and 3.0 mg/kgm of body weight per day (equivalent to approximately 100 p.p.m. in the diet) for the dog."

Attitude of Federal Food and Drug Administration. The Food and Drug Administration, Department of Health, Education and Welfare, in a letter dated October 21, 1958, stated their position in the following words: " . . . As a matter of general policy, we of course take the position that where special additives are used they should not result in the adulteration of foods, drugs, or cosmetics.

"We have had occasion to consider the suitability of cyclohexylamine and morpholine as anticorrosive agents for steam lines where the steam may contact food. We have found that where the steam does not contain more than 10 parts per million of the amines representing the combined and free amount of either, there is no hazard to health."

This letter has been supplemented under date of December 9, 1958, with another, which reads in part " . . . We have not sanctioned the use of any anticorrosive agent in steam which would contact fluid milk.

"We have not sanctioned the use of any ammonia compounds for use in boiler water under the conditions you describe. We would think that such substances would be food additives as described in the Food Additives Amendment and should not be used under conditions whereby they might be introduced into food, except pursuant to an order issued by this Administration, which would, of course, have to be based on a petition filed by some interested person."

The purpose of this discussion was to point out the advantages and disadvantages of direct heating with steam and the obstacles involved. Solutions to problems which would be encountered were suggested on a generalized basis. Each branch of the dairy and food industry would have peculiar problems of their own which would have to be taken care of locally. Water treatments depend upon water conditions and, hence, generalization is all that can be undertaken in a discussion such as this.

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PROBLEMS IN COTTAGE CHEESE PRODUCTION FOR 1959 [1]

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The biggest problem facing the cottage cheese industry in 1959 is the same as it was in 1958—that is, to make the best cottage cheese in the most efficient manner, to get it to the consumer in the shortest time possible with the best flavor, and at a price that will encourage the consumer to eat more cottage cheese than ever before. Although shelf life of cottage cheese has been greatly prolonged, it does not improve with age. Freshness is still a premium factor.

Let us enumerate the important steps, not necessarily in order of importance, that will help achieve the stated objectives. What is the first requirement for high-quality cottage cheese? It is high-quality milk of good flavor and good keeping quality. Need it be Grade A milk? Not necessarily—in some markets it might need to be Grade A, but it does not follow that only Grade A milk is of fine flavor or good keeping quality. Furthermore, let us ask the following questions and give brief answers:

(a) Is Grade A milk safer than ungraded milk? It should be, if Grade A regulations are properly enforced.

(b) Will cottage cheese made from Grade A milk protect the public health better than that made from ungraded milk? It is possible, although properly pasteurized milk is safe unless heat-stable toxins have been produced in contaminated raw milk.

(c) Is Grade A milk lower in bacterial count than ungraded milk? It is probable, but not necessarily so.

(d) Does Grade A milk have a better flavor than ungraded milk? Not necessarily so.

(e) Is Grade A milk more likely to be free from mastitis infection than ungraded milk? Not necessarily so.

(f) Is Grade A milk more likely to be free from antibiotics than ungraded milk? No.

(g) Is Grade A milk more free from sediment than ungraded milk? Not necessarily so.

(h) Is Grade A milk stored at a lower temperature than ungraded milk? Probably, but not always.

(i) Is Grade A milk produced under more sanitary conditions than ungraded milk? Yes, if Grade A regulations are properly enforced.

(j) Is Grade A milk more likely to be free from rancid flavors than ungraded milk? No.

(k) Can you tell by eating cottage cheese whether it is made from Grade A milk or not? No—probably not.

[1] Presented at Dairy Technology Conference, University of Illinois, March 25 and 26, 1959.

The use of poor-quality milk is not being advocated. However, it is true that Grade A milk is not the only kind of milk that can be used for good cottage cheese, because at present a relatively small percentage of cottage cheese is being made from Grade A milk. The consumption of cottage cheese now is at an all-time high. Nevertheless, it is necessary to have milk, regardless of its label, which is of good flavor—free from feed, malty, unclean, rancid, and other off-flavors, that carry over to the cheese curd. It must be free from antibiotic or inhibitory substances; it should be of such bacterial population that after pasteurization it will have a good keeping quality; it should be clean, and it should be free of toxins and pathogenic organisms.

The second requirement is that the milk be of normal composition— free from adulteration. High solids skimmilk of 9.3 to 9.5% is preferred to low solids skimmilk, to secure maximum yields. It is possible to recover 38–39% of T.S. of the skim in cottage cheese with high solids milk, but only 31–33% if low solids milk is used. Maximum profits can be secured only if maximum yields are secured. Fortification of skimmilk is generally practised today, to be assured of high solids. Pasteurization should follow restandardization of solids by nonfat dry milk solids. In other words, powder should not be added to the skimmilk at the vat at time of setting. Standardization of the milk using a lactometer is a good practice. A reading of 37 to 38° lactometer reading is satisfactory. Yield of cheese per 100 lb. skimmilk can be calculated from the formula:

$$\text{Yield} = 4.9 \times [\%\ \text{total solids in skim}] - 29.7.$$

For example: Skimmilk has 9% T.S. Then $[4.9 \times 9] - 29.7 = 14.4$. Yield of dry curd containing 80% moisture should be very close to 14.4 lb./100 lb. skim.

A third requirement is that the skimmilk be properly pasteurized. Although excessively high pasteurization temperatures are used for many dairy products, the minimum temperature that provides for negative phosphatase is best for cottage cheese milk. The most desirable type of body and texture is obtained from normal heat treatment. Lower yields due to shattering can be expected from milk heated above normal pasteurization temperatures, as well as securing cheese having a weak body and texture.

The pasteurized milk should have a low bacterial count if good keeping quality in the cheese is to be expected. Generally speaking, the original milk should have a low bacterial

count and small numbers of heat-resistant bacteria if the pasteurized count is to be low.

The fourth factor of importance is trained personnel and adequate manufacturing facilities. High-quality cottage cheese is a specialized, competitive product that must be made by experts who have proper facilities for doing a satisfactory job. Many dairy companies today are finding it more economical to buy their cottage cheese from companies which specialize in cottage cheese manufacture, than to make it themselves. The cost of milk, cost of labor, cost of new modern equipment, the problems of culture control, antibiotics in milk, bacteriophage, slow sets, lack of adequate refrigeration, poor keeping-quality of cheese, have all been factors in bringing about consolidation or integration of cottage cheese manufacture. Certainly, the trend is for larger and larger cottage cheese operations. Larger plants will install labor-saving and automatic equipment as soon as it is available and it will fit their operating needs.

The fifth factor of importance is culture control. If one is to make a cultured product, but can not maintain cultures, he might as well quit, for he will have to sooner or later. Volumes of literature have been published on starters, and many years have been devoted to research on starters, but as yet we do not have all the answers. Nevertheless, this should not deter us from using the best information we have available on starter care and propagation.

Important steps in maintaining cultures: (a) Secure an active starter culture; (b) have a good milk supply free from antibiotics—tested reconstituted nonfat dry milk solids provides a good culture medium; (c) use proper sterilization procedures for the milk—20 lb. pressure for 10 min., or 190° F. for 1 hr.; (d) maintain proper temperature (70–72° F.) for incubation; (e) transfer in a phage-free atmosphere; (f) incubate for 12 hr.; (g) cool to 40° F. after incubation, (h) maintain supply of frozen cultures or milk inoculated with culture and then kept frozen ready to be incubated.

Temperature control during incubation is probably the most important single factor in maintaining uniformity in cultures.

The sixth factor is know-how in the actual manufacturing procedures. If possible, use a short-time set or some modification of it to reduce growth of contaminants during incubation. Use a minimum amount of starter to give desired acidity in the allotted time. Adjust incubation temperature to 78–86° F., depending on work schedule. Use rennet extract at the rate of 0.6–0.8 ml/1,000 lb. skimmilk or coagulator at equivalent rate, so as to avoid early coagulation by enzyme without acidity. Cut curd at proper titratable acidity, depending on composition of milk and size of curd particles desired. Proper pH may be as high as 4.8 or as low as 4.65, depending on curd size, size of vat, and rate of heating. Acid development during manufacture is desirable and necessary, but excessive development is harmful. Many lots of cheese have become soft and yields have been reduced because excessive acid was developed during the initial stages of heating. An increase of 0.05–0.07% is desirable, and hot water can be used to dilute the acid concentration if necessary.

Careful and controlled agitation is essential during cooking so as to secure uniformity in heating. However, excessive agitation promotes low yields and lack of uniformity in curd size.

Cooking of the curd is extremely important and is a determining factor in the keeping quality of the cheese. Cook at 125 to 135° F. for sufficient time to firm the curd. This may take 30–60 min. Cooking temperature is controlled by pasteurization temperature and acidity. High pasteurization temperature (165° F.—16 sec.) and high acidity (pH 4.5) require high cooking temperature.

After cooking, cool the curd gradually by adding wash water. Use three waters at 80, 60–65, and 35–40° F. Whey is removed from the interior of the curd by the action of the water and the curd is cooled—both are necessary. The water used should be chlorinated or sterilized in some manner, and the third water, particularly, should be adjusted in pH to 4.8–5.0 by the addition of a food-grade acid like hydrochloric or phosphoric. Excessive aroma can be developed in the cheese if citric acid is used. Phenol and medicinal-like flavors can be very troublesome problems in areas where surface water is used. Carbonated beverage plants can not use such water and neither should cottage cheese plants. There should be approximately 20-min. intervals between addition of waters. The final water may remain on the curd 30 to 60 min., depending on how completely the curd is chilled.

Final draining may take 30 to 60 min., depending on the condition of the curd. The vat should be cooled with 32° F. sweet water in the jacket during draining and creaming.

Dressing should be applied to the curd after it has been drained, to maintain its desirable meaty body. The dressing usually constitutes 33–35% of the finished cheese. The cheese contains 0.9–1.0% salt. If the dressing is colder than the curd, it will penetrate into the curd rapidly. Usually, 1 hr. is required for dressing absorption before packaging can be done, if the cheese is packaged from the vat. Otherwise, uniformity in composition can not be maintained. If a known weight of curd is placed in a can containing a known weight of dressing, usually packaging is delayed until the following day. Temperature at packaging should be 50° F. or below.

Sanitation control, although listed as seventh, is of prime importance. Unless excellent sanitation practices are followed, the shelf life may

TABLE 1

Growth of added coliform organisms during incubation, manufacture, and storage of cottage cheese [a]

Stage of manufacture — Lot number	1	2	3	4
Time and temperature of incubation	12 hr. at 72° F.	12 hr. at 72° F.	6¼ hr. at 86° F.	5 hr. at 86° F.
		(No. per ml. or g. of cheese)		
Number organisms added/milliliter milk	500	600	3,500	1,500
Number organisms at cutting curd (pH 4.6)	< 1,000,000	46,000	23,000	(pH 4.8) 16,000
Curd after reaching 105° F.	.....	240,000	.....	900,000
Whey after reaching 105° F.	.....	2,600	.....	7,000
Curd after reaching 110° F. (pH 4.55)	140,000	114,000	24,000	130,000
Whey after reaching 110° F.	0 in 10^3 dilution	1,400	6,000	5,000
Curd after reaching 120° F.	8,000	80	100,000	(pH 4.7) 110,000
Whey after reaching 120° F.	0 in 10^3 dilution	1	29	4,000
Curd held 30 min. at 120° F.	0 (1–10)	0 (1–2 dilution)	25 (1–2)	110,000
Whey held 30 min. at 120° F.	0 (1–10)	0 (1 ml.)	0 (3 ml.)	200
Curd after reaching 130° F.	0 (1–2)	0 (1–2 dilution)	1 (1–2 dilution)	60
Whey after reaching 130° F.	0 (2 ml.)	0 (1 ml.)	0 (3 ml.)	0 (in 3 ml.)
Dressing	0 (1–2)	0 (1 ml.)	3 (10/3 ml.)	0 (in 2 ml.)
Dry curd after washing (3 hr. after cutting)	0 (1–2)	(1–2)	0 (1–2 dilution)	0 (3 ml. 1–2)
Creamed cottage cheese (pH 5.2–5.3)	0 (1–2)	0 (1–2)	1 (1–2 dilution)	0 (5 ml. 1–2)
After 2 days at 60–62° F.	.....		200,000	4 (1–2)
After 4 days at 60–62° F.	.....	60,000,000	55,000,000	
After 7 days at 60–62° F.	> 19,000,000	40,000,000 (pH 4.78)	43,000,000	1,300,000 (14 days pH 4.95)
After 4 days at 48–50° F.	.....	20,000		
After 7 days at 48–50° F.	.....	2,000,000 (pH 5.23)		
After 2 days at 38–40° F.			4 (1–2 dilution)	0 (1–2)
After 4 days at 38–40° F.		0 (1–2 dilution)	6 (1–2)	
After 7 days at 38–40° F.		8 (1–2 dilution)	4 (1–2)	
After 14 days at 38–40° F.			6 (1–2)	0 (3 ml.–1/10)
After 14 days at 38–40° F. (dry curd)		0 in (1–100 dilution)		
After 21 days at 38–40° F.	2 (1–2) good flavor (pH 5.28)		1 (2 ml.–1/10)	0 (3 ml.–1/10) (slightly bitter)

[a] Acknowledgment is given to Yasuo Azuma for making the bacteriological analyses.

be only two instead of 21 days. The following factors are important in determining shelf life of cottage cheese: (a) Original bacterial count of milk and the number surviving pasteurization; (b) contamination occurring following pasteurization—pipes, vats, agitators, etc.; (c) growth occurring during period of incubation; (d) number or proportion of bacteria killed during cooking of the curd; (e) contamination following cooking, which is added by equipment and wash water; (f) number and kinds of organisms added by the dressing; (g) number and kinds of organisms added by storage cans, agitators, shovels, scoops, pipes, pumps, and packaging machine, (h) temperature of cheese at packaging and temperature maintained during storage.

In some areas, there are public health ordinances requiring that cottage cheese have less than ten coliform organisms and less than ten yeasts and molds per gram of cheese. To study the growth characteristics, as well as their resistance or lack of resistance to the cheese-making process, four lots of cheese were made, to which coliform organisms were added to the pasteurized milk. These organisms were then followed throughout the process of manufacture and storage of the cheese. The results of this study are given in Table 1 and reveal the following:

(a) In spite of heavy inoculation, growth during incubation was not great enough to cause an off-flavor or a gassy, pin-hole cheese to be produced.

(b) These organisms are almost completely eliminated by the cooking at 130° F. It is possible to get zero count/gram cheese in a (1-2 dilution). Organisms are killed more quickly in whey than in curd.

(c) Rate of growth of those coliforms surviving cooking is rapid at 60–62° F., is less rapid at 48–50° F., and at 40° F. is almost negligible. In Lot 1, after 21 days at 38–40° F., the coliform count was 2 (1-2 dilution), but at 60° F. it was more than 10,000,000 in seven days.

(d) Growth of yeast and molds and psychrophiles was not a problem at 40° F., but would cause concern at 48–50° F. or 60° F.

(e) It is possible to secure a zero count in a (1-2) or 1-10 dilution on fresh cheese under the following conditions: 1. Care is exercised during the creaming and packaging of the cheese; 2. cheese is stored at 40° F. or below, 3. sample is secured shortly after packaging.

What is the significance of coliform organisms in cottage cheese? Coliform organisms in cottage cheese have no public health significance other than indicating the sanitary condition of the equipment, the sanitary technique employed during manufacture, and perhaps the sanitary knowledge of the cheese-maker.

Sterilization of all equipment used in conjunction with cottage cheese manufacture is essential. Utensils used in the manufacture of cheese should be taken directly from a sterilizing tank at the time of use and again immersed in the sterilizing medium when not in use. This procedure, of course, applies during the manufacturing operation.

The cheese-maker should be a careful operator. He should wear clean clothes, have a chlorine solution available for washing his hands when he handles paddles, scoops, etc. Single-service towels should be provided.

TABLE 2

Yeasts, molds, and psychrophiles per gram cheese

Conditions	Yeasts	Molds	Psychrophiles
		Lot 1	
Fresh cheese	0 (1–2)	0 (1–2)	0 (1–2)
After 7 days at 60° F.	12,000	50	Less than 10
		Lot 2	
Fresh cheese	0	0	0
After 7 days at 60° F.	15,000	0	8,600,000
After 5 days at 48–50° F.	3 (1–2)	3 (1–2)	
After 7 days at 48–50° F.			6,300,000
After 5 days at 38–40° F.	0 (1–2)		Less than 10
		Lot 3	
Fresh cheese	0 (1–2)	0 (1–2)	Less than 10
After 7 days at 38–40° F.	.....	.....	.....
After 14 days at 38–40° F.	0 (1–2)	0 (1–2)	5,000
After 21 days at 38–40° F.	1,000	66 (1–2)	Less than 5,000
		Lot 4	
Fresh cheese	0 (1–2)	0 (1–2)	Less than 10
After 7 days at 38–40° F.	.....	.....	.....
After 14 days at 38–40° F.	0 (3 ml.–1/10)	0 (3 ml.–1/10)	Less than 10,000
After 21 days at 38–40° F.	0	20 (2 ml.–1/10)	Less than 10,000

The outside of the vat should be kept clean, otherwise contamination can occur in the following cycle—outside of cheese vat→clothes→hands→utensils→cheese.

The following is a list of important sources of contamination by organisms that cause spoilage in cottage cheese:

Psychrophilic Organisms

1. Wash water
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Pipes
8. Filler or packaging equipment
9. Curd scoops
10. Vegetables used to flavor cottage cheese
11. Other equipment or products, not sterilized, used in cottage cheese manufacture
12. Direct contamination by cheese-maker

Coliform Organisms

1. Pipes
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Filler or packaging equipment
8. Curd scoops
9. Vegetables used to flavor cottage cheese
10. Other equipment not sterilized, or products not handled in approved manner
11. Direct contamination by cheese-maker

Yeasts and Molds

1. Pipes
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Filler or packaging equipment
8. Curd scoops
9. Cartons and lids
10. Vegetables used to flavor cottage cheese
11. Other equipment not sterilized, or products not handled in a sanitary manner
12. Direct contamination by cheese-maker
13. Air-borne contamination

Cottage cheese is an excellent food. It is one of our cheapest and best sources of proteins. It is looked on very favorably by the medical profession at the present time. The public is eating more cottage cheese today than ever before. There have been no epidemics traced to cottage cheese made from pasteurized milk. There apparently have been no food-poisoning cases traced to cottage cheese. The dairy industry can be proud of what it has achieved in improving the quality of cottage cheese. This has been brought about by competitive forces within the industry and by the increased level of understanding of sanitation and public health by dairy plant operators. The consumer confidence and acceptance that the dairy industry has, is certainly due in part to the confidence that the public has in public health officials. Let us continue to do those things which will continue to give the consumer the best cottage cheese at a fair and reasonable cost.

BOOK REVIEWS

THE HANDBOOK OF FEEDSTUFFS. Rudolph Seiden and R. H. Pfander. Springer Publishing Company, Inc., New York, 1957.

This book is a very abbreviated encyclopedia covering the grains, the minerals, and the plant and animal by-product feeds. In addition, brief botanical descriptions of most of the plants which are fed in the United States are provided, and the pests and diseases affecting them are mentioned along with suggested control measures.

Pharmaceuticals and feed additives are listed and their use briefly described. Terms commonly used in feeding are defined very simply.

Considering the tremendously broad subject matter areas encompassed in the book's subtitle, Production, Formulation and Medication of Feedstuffs, it is easy to comprehend the somewhat inadequate explanation found in the text.

This book can serve as a reference for workers in the feedstuffs industry and possibly for others concerned in the general area of feeding.

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MEAT HYGIENE. A. R. Miller. Second Edition. Lea & Febiger, Philadelphia. 557 pages.

This book is intended as a text for students of meat hygiene or for those who are engaged in the inspection of meat and poultry animals. It is, however, sufficiently comprehensive and authoritative to be a desirable reference work for all who are concerned with the processing of meat and poultry products.

The varied material of this book is presented in 17 chapters. The first two chapters establish a background for the remainder of the book by discussing History and Elements of Meat Hygiene, the latter chapter being rather brief, for establishing a proper perspective on the importance of meat hygiene. Chapters 3 and 4 describe the techniques and the pathology involved in the ante-mortem and post-mortem inspection of both meat animals and poultry and, together with Chapter 8 on Facilities for Inspection, adequately take care of this phase of the subject matter. The problem of trichinae in pork products is presented in Chapter 5; the discussion is supplemented with the reproduction of important reports on trichinosis and summaries of several important court cases which involved infected pork meat. Chapter 6, entitled Physical and Chemical Characteristics of Meat and Principal Organs, provides a general background in the histology and chemistry of meat.

The very important problem of sanitation in plant operation is handled in Chapter 7, one of the longer chapters of the book. The first half of this chapter deals with fundamentals of sanitation, including water supply, waste disposal, insect and rodent control, detergents, and disinfectants; whereas, the remainder largely describes equipment but does include some meat technology. Additional technology is supplied in Chapters 9 and 10. Chapter 9, entitled Materials Added to Meats, of necessity must be somewhat encyclopedic in nature, but one questions the necessity of some of the historical and descriptive detail such as is used to describe the various sugar preparations. In Chapter 10, the author provides a concise account of the handling of carcass meat, meat processing, and the preparation of a variety of meat products. Meat and poultry grading is outlined in Chapter 11. Chapter 12, Adulteration and Misrepresentation, Chapter 13, Deteriorative Changes in Meat, and the final chapter (17) Organized Meat Hygiene Control supplement the earlier discussion on sanitation and emphasize the legal aspects of the inspection programs.

The three new chapters on Food Poisoning, Chemical Additives, and Ionizing Radiation are brief but helpful in providing a general background on these subjects, and are desirable towards completing this discussion on food or meat hygiene.

The book is well written. The illustrations and diagrams are clear and well chosen. It is relatively free of typographical errors or other minor mistakes, although this reviewer was somewhat chagrined to read a three-paragraph discussion on *Escherichia coli* under the printed subheading *Enterococcus*.

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BREEDING 10,000,000 CATTLE. Production Division, Milk Marketing Board, Thomas Ditton, Surrey, England.

This book is a synoptic history of the artificial insemination service operated by the Milk Marketing Board. From a rather modest beginning in 1944-45, in which year approximately 2,600 cows were bred, the service grew rapidly to 1957-58, in which year approximately 1,399,000 cows were bred. At the end of December, 1958, the Board's A.I. service had bred 10,000,000 cows; thus, the title of the book.

In addition to this rapid growth, the A.I. service of the Milk Marketing Board should be proud of its committee organization, its methods of choosing and testing bulls, its plan of rearing bull calves, and its insemination fee of one pound. The material on selection pro-

cesses and rearing of young bulls, pages 7-13, inclusive, should be of interest to all A.I. organizations.

A.I. service is available from 15 breeds, of which four are dairy, four dual purpose, and seven beef breeds. During the 14 yr., there have been major changes in the numbers bred to bulls of the various breeds. On pages 20 and 21 are listed the numbers of cows bred per year to the various breeds of bulls. Two interesting facts about this table are the early and rapid increase in the numbers bred to Friesian bulls and the later and almost as rapid increases in the numbers bred to Aberdeen Angus and Hereford bulls. It is suggested that these result from a wish to obtain higher milk yields and an attempt to breed for a cross that will meet demand either for size or quality in producing beef. In more recent years, the A.I. service has emphasized the production of beef from the dairy herd.

The history of the A.I. service of the Milk Marketing Board is a story of success, and in the book, Breeding 10,000,000 Cattle, that story is told briefly and well.

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PHOTOMETRIC MILK FAT DETERMINATION[1]

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SUMMARY

The theoretical consideration and practical application of a photometric procedure for milk fat determination are presented. The interfering turbidity due to casein micelles is eliminated chemically by use of a calcium chelating agent. Coherent scattering is eliminated by a fivefold dilution of milk samples. Using a favorable wave length of light, in the visible range, turbidity is measured as a function of average globule size for the size range of fat in homogenized milk. The two characteristics of the milk fat emulsion which then remain unalterably to influence the total scattering are fat content and average globule size. Two independent photometric measurements are required to solve for the two variables simultaneously. The parallel transmitted and the total transmitted light through a thin layer of the prepared sample constitute two independently variable intensities. Mathematical development of the problem led to two linear equations from which a nomogram can be constructed to read both fat content and average particle size. From photometric readings on known samples, a nomogram was drawn and the two characteristics read for a number of commercial milk samples. The results agree well with the Babcock comparative values. Applicability of the present method to other dairy products is discussed.

A milk fat test which is simple, accurate, rapid, and free of personal factors has long been desired in the dairy industry. Such a method would most likely be of a physical rather than chemical nature. Many factors which influence and limit the determination of milk fat by photometric means must be considered, to arrive at a procedure which can be used routinely. Milk viewed as a sample emulsion for light-scattering studies is a complex product and does not lend itself easily to such studies. A truly simple and practical test must have built into it the elimination of as many of the variables as possible and yet not be limited too narrowly in application as regards fat content and type of product. Accuracy and precision of the test must, of course, be comparable to those of existing methods.

Turbidity measurement has been a standard analytical procedure for half a century, and it is natural that many workers have made attempts with varying degrees of success to adapt such a procedure to determine either fat content or homogenization efficiency in dairy products. One of the factors which complicates the method is the disturbing influence of casein micelles. Some workers have tried to circumvent this by extracting the fat to form a new emulsion free of casein turbidity (Bloor, 4; Fedotov, 9; Goiffon, 10; and Heinemann, Baldi, and Parker, 13). Others add alkali or ammonia to milk to destroy the micelles (Ashworth, 1; Brio and Vlodavets, 6; and Deackoff and Rees, 8). The casein micelles are known to be 0.1 μ in diameter, whereas fat globules of homogenized milk average 2 μ. Thus, if light of 0.7 μ or longer wave length is used, no scattering due to the micelles can be detected. A third way of eliminating the influence of micelles, then, is to do so optically, by selecting light of sufficiently long wave lengths, as

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[1] Reprints available for 90 days after publication of this paper. For prices see page 1434 of this issue.

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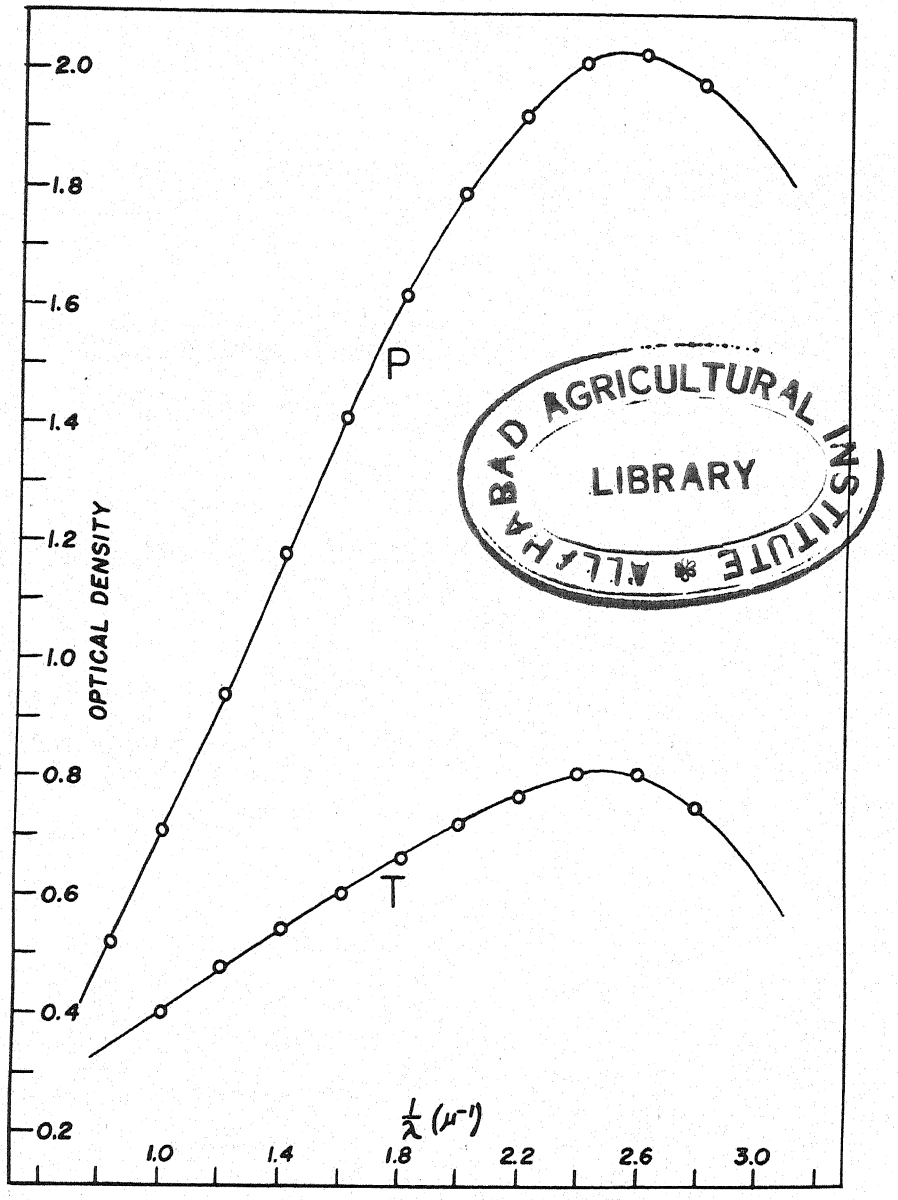
reported by Deackoff and Rees (8), Goulden (11), and Leviton and Haller (17). Another factor of considerable importance which has been reported in a number of articles is the influence of the heterodispersity of the fat emulsion and, as a corollary, the shape of the size distribution curve of the globules, as shown by Borg (5), Lagoni and Merten (16), and Schneck and Mengebier (25). Important contributions to the study of milk turbidity, which should also be noted, have been made by Burgess and Herrington (7) and very recently by Goulden (11) in a series of papers. The difficulties brought forward here, and other factors as well, have been dealt with in a fresh approach to the problem, in order to arrive at a practical solution.

THEORETICAL BACKGROUND

Two important parameters.—It is not of importance here to discuss the theory of light scattering in detail.[1] It shall only be mentioned that two parameters are of great theoretical importance, namely, $m = \frac{n_1}{n_2}$, which is the value of the refractive index of the particle relative to the refractive index of the medium, and $\alpha = 2\pi r/\lambda$, where r is the radius of the globule and λ is the wave length of light. The same two parameters are, consequently, also of importance for any practical application of light scattering. The parameter m should vary only within a narrow range, that is, the refractive index of the lipid phase as well as of the milk serum should not vary much from animal to animal or from season to season. The variations relative to the serum are eliminated as a factor in the final procedure by a fivefold dilution of the milk. This has the compensating effect of narrowing the range of the natural variations. The refractive index range of the lipid phase, on the other hand, should be rather narrow, because a similar compensation can not be applied. It is demonstrated later in the paper that the natural variations are narrow enough in the lipid phase so that errors from this source can be disregarded.

The second parameter $\alpha = 2\pi r/\lambda$ is of the utmost importance for this method. If α is plotted against the total scattering or scattering in any specific direction, the result is an oscillating curve—here called an alpha curve. These curves consist, practically speaking, of rather large linear sections connected by short, curved ones. The value of α can be varied systematically by varying r while keeping λ constant, or vice versa. Two such curves were obtained by varying λ. To a homogenized milk sample (2% fat) were added alkali and a chelating agent, to destroy the casein micelles. A collimated beam of light was sent through a thin layer of the sample and both the total transmitted light *(T)* and the parallel transmitted light *(P)* were measured by a procedure which will be described in detail. The measurements were repeated with light of different wave length. Figure 1 depicts the two curves obtained from these measurements. The characteristic appearance of the α curve can be visualized from these curve sections, even if they show only one maximum. A fully developed α curve calculated for

[1] Presentation of the theory is found in papers by Oster (20), Sinclair and La Mer (27), and Van de Hulst (14).



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FIG. 1. Alpha functions obtained by varying wave length of light.

the milk system is presented in a paper by Goulden (11), reproduced in Figure 2. The ordinate value K is the scattering coefficient, which depends upon a number of factors such as a, m, etc. Other calculated or experimental a curves are presented in papers by Oster (20), Sinclair and La Mer (27), Lewis and Lothian (18), and Tabibian and Heller (29).

Importance of independent scattering. When casein turbidity is eliminated, only fat globules will scatter light. The light scattered by each fat globule will

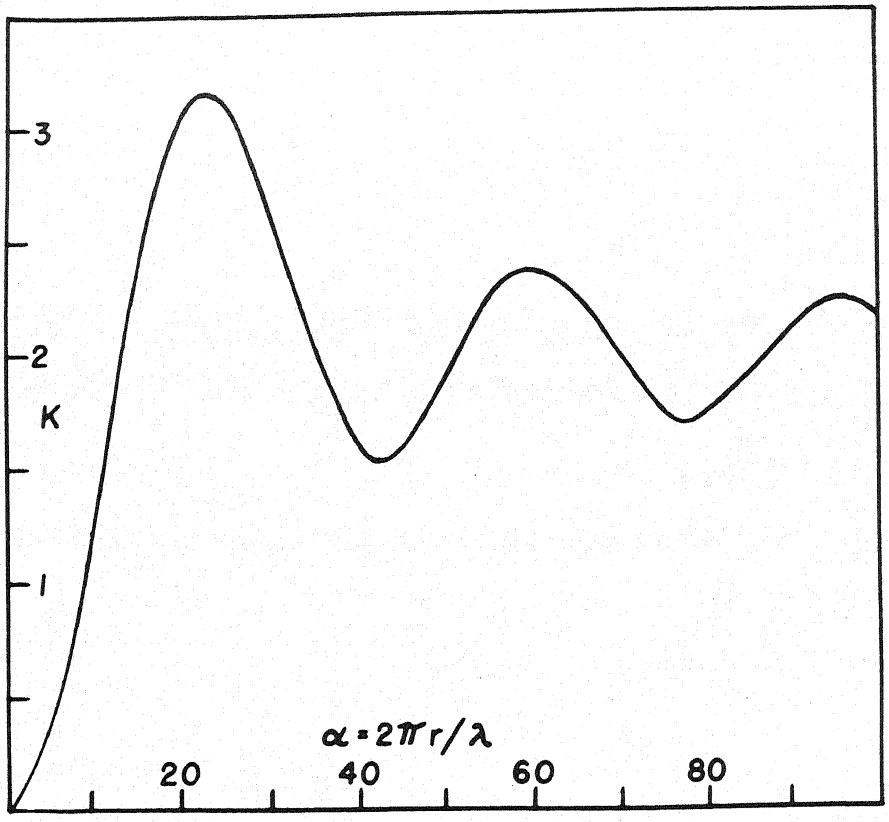


FIG. 2. Alpha function for milk fat after Goulden (11).

be independent of the scattering by other fat globules present, when the milk is sufficiently diluted. The total scattering is then simply the sum of the scattering from each contributor. Only two factors will, under these circumstances, influence the scattering of light; namely, fat percentage and average size of the globules and, consequently, two independent measurements are required to solve for one or both of these quantities. Coherent scattering would be a factor in the measurements if the concentration of fat is higher than a certain characteristic value, and becomes of increased importance the higher the fat concentration. The total scattering would, then, depend not only on the two factors just mentioned but also on a third one—the globule size distribution curve. A practical photometric method for fat determination in milk could under these conditions hardly be developed, due to the increased complexity of the problem.

Derivation of equations. Assuming for the moment that the scattering is a function of the average size of the fat globules and the fat per cent only, the mathematical treatment of the experimental data is rather simple. When a collimated beam of light of intensity I_o is incident to a thin layer of milk, a part, B_w, of the incident light will be scattered in the backward direction; another part, F_w, is scattered in the forward direction, and a third part, P, will pass through

unaltered. These quantities are interrelated by a simple equation, when there is no light absorption.

$$I_o = B_w + F_w + P \tag{1}$$

Any two of these quantities are independent of each other. We have chosen to measure the quantities P and B_w, the latter obtained indirectly by measuring the total transmitted light which is equal to $I_o - B_w$. It is assumed that the layer of milk to be measured is so thin that the attenuation equation (Lambert-Beers Law) can be applied. In other words, no multiple scattering is present. Thus, we have

$$\log \frac{I_o}{T} = \mathrm{A} \cdot \% \text{ fat} \tag{2}$$

and

$$\log \frac{I_o}{P} = \mathrm{B} \cdot \% \text{ fat} \tag{3}$$

These logarithmic expressions are known as the optical densities. The coefficients A and B depend on the thickness of the milk layer and the average globule size. Differentiating the globules into p groups according to size, we have

$$\log \frac{I_o}{T} = \sum_1^p A_x \%_x \tag{4}$$

$$\log \frac{I_o}{P} = \sum_1^p B_x \%_x \tag{5}$$

We have, furthermore,

$$\sum_1^p \%_x = \%_t \text{ (total fat per cent)} \tag{6}$$

It can be visualized that there exists a milk of uniform globule size and with the same fat content as a nonuniform milk producing the same amount of total transmitted light. This can be expressed as follows:

$$\log \frac{I_o}{T} = \sum_1^p A_x \%_x = A_u \%_t \tag{7}$$

A similar expression can be written for parallel transmitted light. The two respective uniform milks are not necessarily identical, but become so if the coefficients are linear functions of the fat globule radii. This is the case if such conditions are chosen that for a selected wave length of light the range of globule sizes of the samples falls within a linear section of the α-curve. We have then,

$A = ar + b$ and $B = cr + d$. By inserting these expressions into Equation 7, followed by reduction of the terms, the following relationship is found:

$$r_u = \frac{\sum_1^p r_x \%_x}{\%_t} \tag{8}$$

where r_u is the globule radius of the uniform milks. As the coefficients A and B disappear from the equations, and as the same treatment applied to parallel transmitted light leads to the same equation, it follows that the two uniform milks must be identical. We have, thus

$$\log \frac{I_o}{T} = (ar_u + b)\ \%_t \tag{9}$$

and

$$\log \frac{I_o}{P} = (cr_u + d)\ \%_t \tag{10}$$

By eliminating the term r_u we have

$$\log \frac{I_o}{P} = \frac{c}{a} \log \frac{I_o}{T} + (d - \frac{bc}{a})\ \%_t \tag{11}$$

Elimination of the term $\%_t$ gives the following equation

$$\log \frac{I_o}{P} = \frac{cr_u + d}{ar_u + b} \log \frac{I_o}{T} \tag{12}$$

Equation (11) describes a family of parallel and equidistant lines for equal increments of fat content and Equation (12), a family of lines whose slopes depend on the value of r_u. Experimental data confirm Equations (11) and (12). A nomogram can be constructed from those equations or from the corresponding experimental data. From such a nomogram, the fat per cent can be read, as well as the average globule size or an equivalent expression.

Demonstration of presence or absence of coherent scattering. The mathematical treatment derived above is correct only under the condition that there is no coherent scattering and no multiple scattering. The latter factor restricts measurements to fat concentrations where Lambert-Beers law is valid. Coherent scattering is naturally more pronounced the higher the fat concentration and will, under a certain concentration, be of insignificant influence. The following is a good test for presence or absence of coherent scattering. If $\log \frac{I_o}{P}$ and $\log \frac{I_o}{T}$ are plotted against each other for milks of the same fat content but different fat globule size distribution curves, one would find the points distributed along a

smooth, nearly linear curve, if the globule size distribution had little or no effect. If, on the other hand, this factor is of importance, great deviations can be expected and would reflect on the accuracy of fat determination when comparing milks with globule size distributions other than those of the calibration curves. That this is the case for nondiluted milk is clearly illustrated in Figure 3. The points corresponding to mixtures, of milks homogenized at different pressures, do not fit on the calibration curves. The result of this test indicated to us the reason milk fat could be determined satisfactorily when all samples were processed through the same homogenizer, but consistent deviations from calibration curves were found when other homogenizers were used. The casein turbidity in these earlier experiments was eliminated by making the photometric measurements using light of 1-μ wave length.

A similar test was repeated for milk diluted one to five and measured with light of 0.6-μ wave length. This corresponds to the final choice of dilution and light. Dilution of milk samples being a required condition in the final measuring procedure, the disturbing influence of casein micelles was eliminated chemically by incorporating a chelating agent in the diluting solution. This permitted the selection of a favorable wave length from a much wider range. The results of this experiment are depicted in Figure 4. No odd globule size distribution curve, as obtained from mixtures of milks homogenized at different pressures, gave points which deviate appreciably from the calibration curve. No deviations were found for milks processed by different homogenizers, either, as will be demonstrated in the experimental section. A calculation shows that the average distance between the fat globules of the diluted samples is about four times their radii, which is in good agreement with the conditions given by Van de Hulst (14, page 5), who states that a distance of three times the radius of the scattering particles is sufficient to insure independent scattering.

INSTRUMENTATION

Spectrophotometer. The Beckman Model DU spectrophotometer was very useful for the development of this method. The total transmitted light component, T, was measured with the photocell compartment adjacent to the sample cell, and the parallel light component, P, by removing the photocell compartment 12 cm. along the light-beam path. Movement was accomplished by sliding the compartment along a pair of parallel rods. The movement area was enclosed in a light-tight box. A water jacket, kept at 40° C. by an electrical immersion rod heater, was attached to the instrument mounting block, so that all samples were measured at the same temperature.

Microcell. A specially made sample cell was required for the photometric measurements because sample light paths worked with were mostly under 1 mm. A stainless steel cell was obtained[2] with inlet and outlet tubes which allow gravity filling and flushing without removing it from the instrument. The cell as shown in Figure 5, mounted in its cell holder, was fitted with Pyrex glass windows

[2] Herman Ruf, Northern Tool and Instrument Co., 162-38 Pidgeon Meadow Road, Flushing 58, New York.

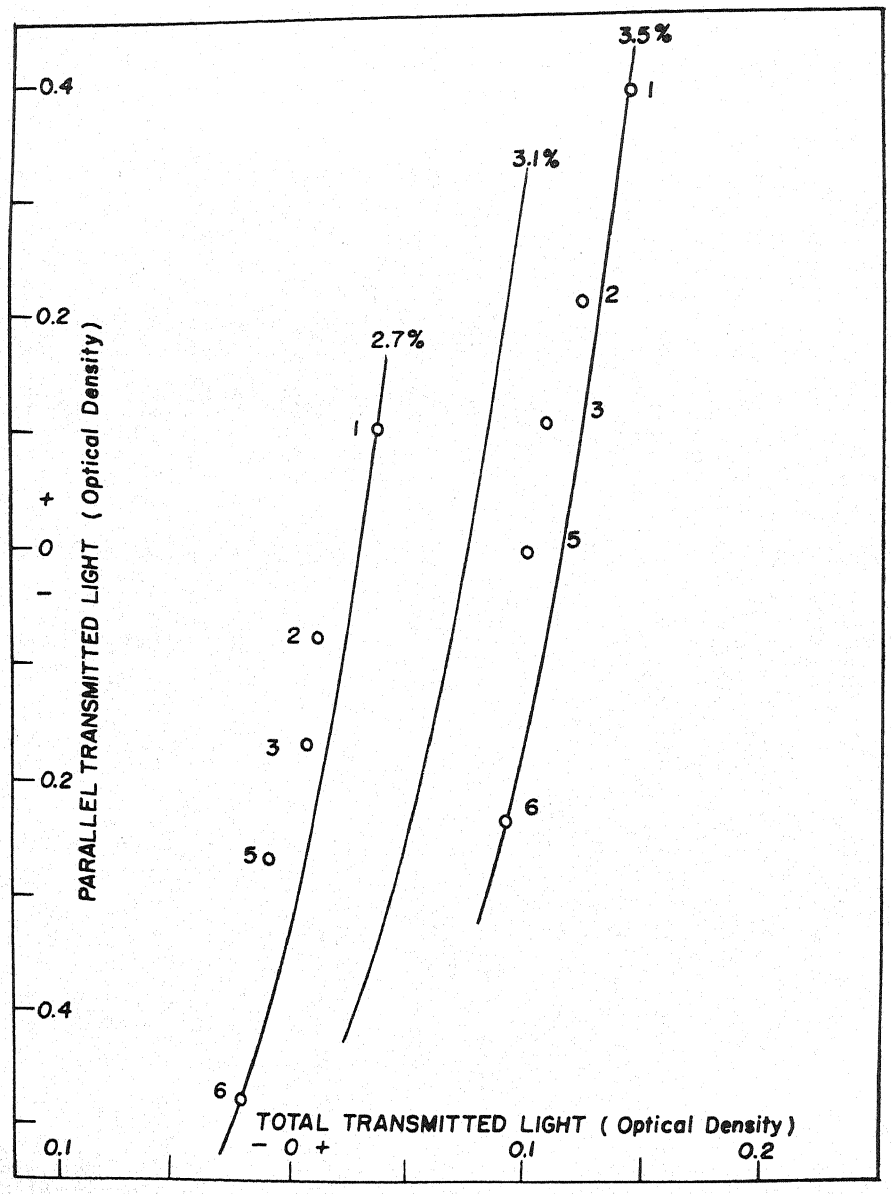


FIG. 3. Graphical demonstration of presence of coherent scattering.

1. Sample homogenized at 1,500 p.s.i.
2. Mixture, two parts sample homogenized 1,500 p.s.i., and one part sample homogenized 3,800 p.s.i.
3. Mixture, one part sample homogenized 1,500 p.s.i., and one part sample homogenized 3,800 p.s.i.
4. Mixture, equal parts of samples homogenized 1,500, 2,400, 3,200, and 3,800 p.s.i.
5. Mixture, one part sample homogenized 1,500 p.s.i., and two parts sample homogenized 3,800.
6. Sample homogenized at 3,800 p.s.i.

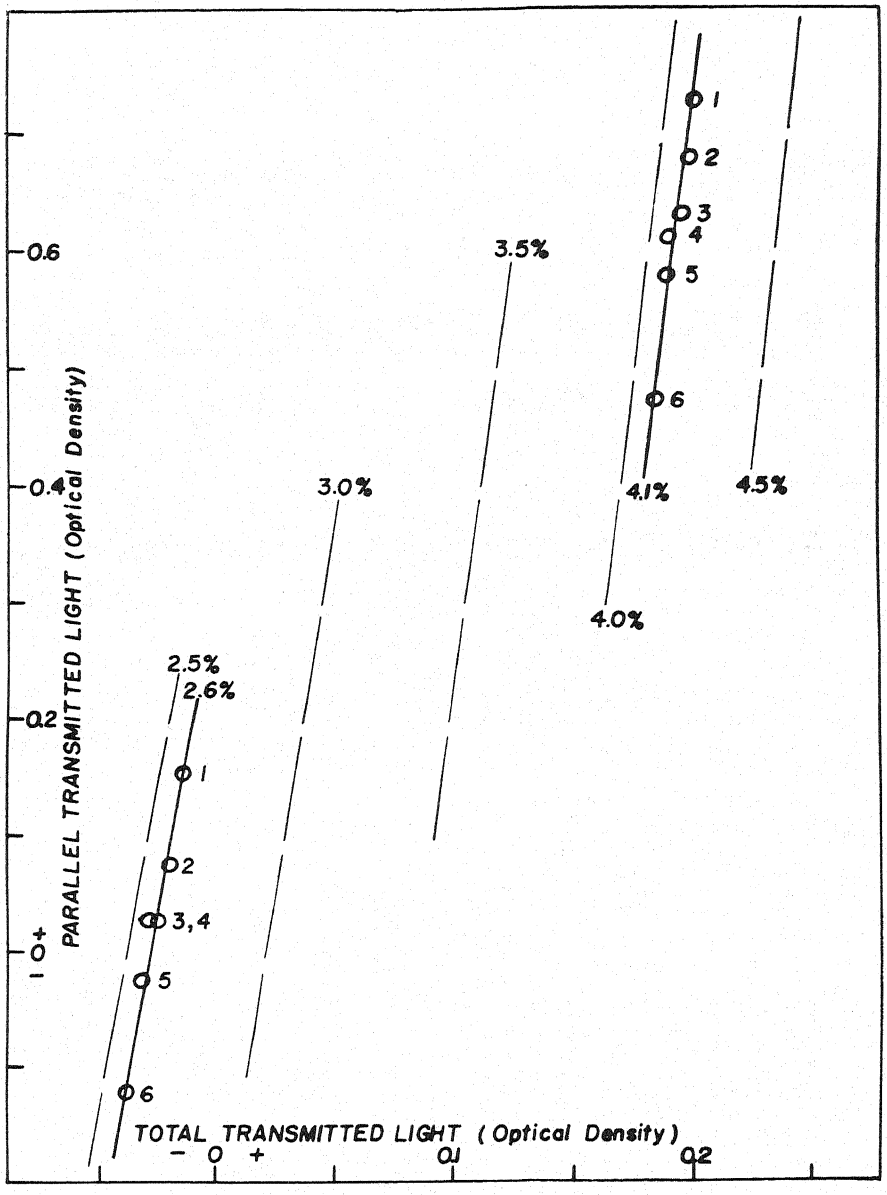


FIG. 4. Graphical demonstration of absence of coherent scattering.

1. Sample homogenized at 1,500 p.s.i.
2. Mixture, two parts sample homogenized 1,500 p.s.i., and one part sample homogenized 3,800 p.s.i.
3. Mixture, one part sample homogenized 1,500 p.s.i., and one part sample homogenized 3,800 p.s.i.
4. Mixture, equal parts of samples homogenized 1,500, 2,400, 3,200, and 3,800 p.s.i.
5. Mixture, one part sample homogenized 1,500 p.s.i., and two parts sample homogenized 3,800.
6. Sample homogenized at 3,800 p.s.i.

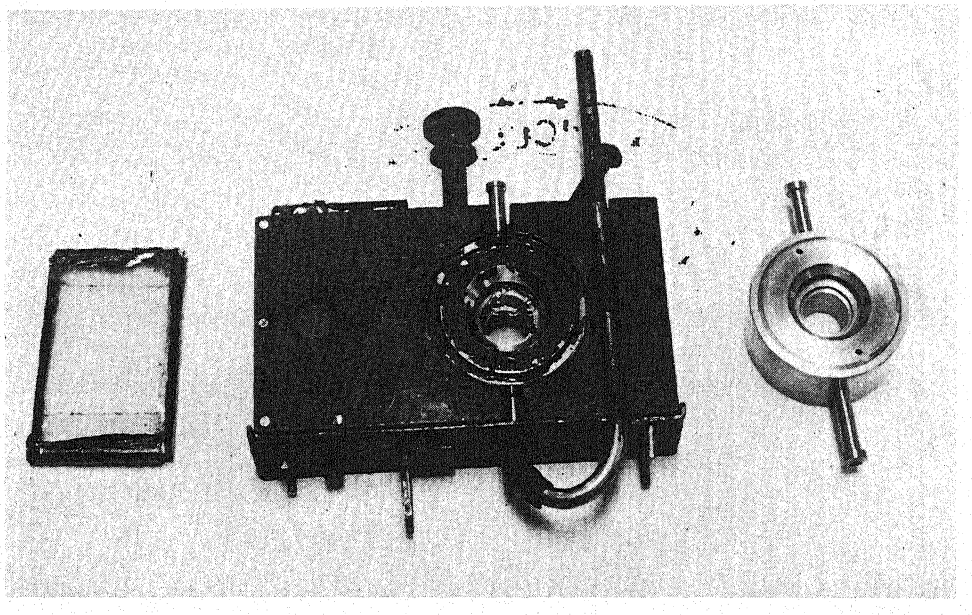


FIG. 5. Cell holder with reference standard and microcell assembled and separate.

with an incident light area 8 mm. in diameter. A channel concentric with the rear window served to lead any air bubbles harmlessly through the cell. Its volume for 500-μ spacing between windows is 0.1 ml.

Milk glass standard. To measure optical density of P or T with reference to an air-light path without a decade selector, the sample light path would need be extremely small or dilution very high. As an alternative, a rectangle of flashed-on type milk glass was selected for a stable reference standard, so that density could be read on the instrument within the range of zero to 0.700 unit. The appropriate standard was selected from a series made by polishing off amounts of the flashed-on layer until various rectangles of the glass read, in turn, 10, 25, 50, and 70% total transmission at 1-μ wave length. It simplified matters to use a milk glass type standard rather than a neutral density filter, so that both P and T could be read in reference to the same standard.

Bench homogenizer. A small, manually operated homogenizer was designed and constructed at our laboratories to process nonhomogenized samples. Shown in Figure 6, it embodied a spring-loaded valve with its tension acting against a fixed orifice plate. Torque applied to the hand lever forced 5 ml. of milk through with each forward stroke. The feed cup check valve permitted the pressure chamber to refill on each back stroke. Valve spring tension could be varied at will for a homogenization pressure range of zero to 4,000 p.s.i., calibrated by hydraulic pressure gauge. The action on the forward stroke was smooth, with constant pressure which gave a uniform homogenization. A built-in electrical heater and thermostat control kept the aluminum jacket at a chosen temperature. The homogenizer disassembled easily by removing six allen-head bolts.

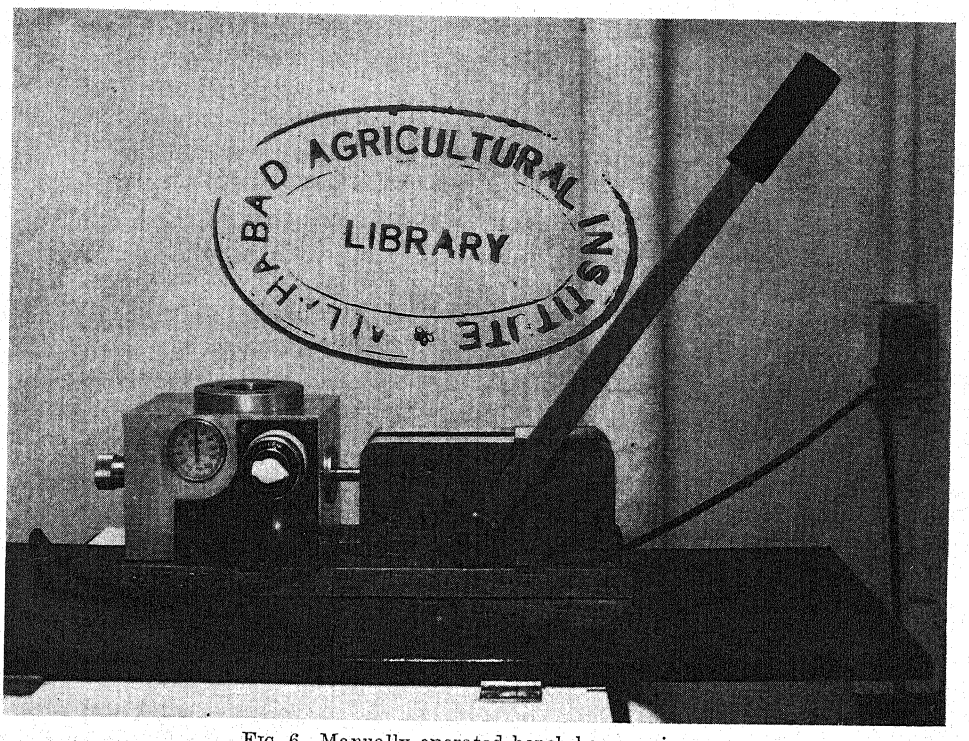


FIG. 6. Manually operated bench homogenizer.

MEASURING CONDITIONS

Homogenized milk. Homogenization narrows the fat globule size range so that the range falls on a favorable section of the alpha curves discussed previously. Neither a monodisperse milk fat emulsion nor a reproducible size distribution is obtainable by homogenization. If it were readily feasible, a single photometric measurement would be directly proportional to fat content. The residual fat in skimmilk meets this requirement to some extent. This was taken advantage of by Pisecky (21), who reported a turbidimetric method for determining fat in skimmilk.

Sample temperature and history. Photometric readings of samples with the fat solidified differed consistently and reproducibly from those with the fat melted. The choice to measure all samples after having been prewarmed to 40° C. was made because it was much less time-consuming than if they were to be refrigerated for a required minimum of several hours. It was also safer to avoid fogging of the microcell windows by samples taken directly from cold storage. The heat treatment history of milk, such as pasteurization by either the holding or flash method and even sterilization at 100° C. for 1 hr., had no effect on the photometric readings.

Sample light path. It is usual procedure to either decrease the sample light path or increase dilution to overcome multiple or coherent scattering, respectively, and maintain reading sensitivity where Beer's law is valid. The importance of

this in the development of the procedure was discussed earlier. In the practical choice of a light path for whole milk, it was seen that when either the P or T optical density readings of a series of samples homogenized at the same pressure were plotted against fat content a curve with a straight line section was obtained. This section represented fat content range where measurement was made with the same sensitivity. Beyond this range, the curve gradually changed its slope as multiple scattering became evident, until there was no further change of reading and, consequently, no sensitivity for increasing fat content. Readings of samples of a given fat content range were made, using different sample light paths. From these a family of curves were drawn where the longer the light path was, the steeper the slope of the straight line section. However, the steeper the slope, the narrower was the range of fat content corresponding to the straight line section. The optimum sample light path of 500 μ for 2—5% milk fat (the fat content after dilution was actually 0.4–1.0%) was, therefore, a compromise between the entire range falling on a straight line section and steepness of slope. A family of straight lines with a common intercept at zero fat was also then produced for a series of homogenization pressures.

Diluting solution. Primarily, milk samples were diluted to eliminate coherent scattering, with the consequence that the globule size distribution curve factor was done away with. It can be visualized that other advantages of a practical nature in favor of dilution are: elimination of the disturbing window effect described by the writers (12); reduction of serum refractive index difference between samples; increase of refractive index difference between milk fat and suspending medium and, of lesser importance, use of longer sample light path so that tolerance of the dimension between windows is not as critical. Dilution of milk with water brought about casein micelle instability which influences the optical properties, as has been investigated by Lagoni and Merten (16). Use of various buffers of different concentrations and solutions of calcium salts did not overcome these unbalances. Reagents such as sodium citrate, sodium oxalate, sodium hydroxide, and disodium Versene (disodium ethylenediamine tetraacetate) were then tried. Versene gave the most satisfactory results for the relatively small amount required. Also, its action in disrupting the calcium-casein complex to form chelated calcium and solubilizing casein to form sodium caseinate is practically instantaneous. A solution consisting of 0.325% Versene, 0.125% Tween 20[3] emulsifier and adjusted to pH 10 with sodium hydroxide formed dilutions which had excellent stability. Emulsifier was incorporated in the solution to prevent fat globule clumping. Dilutions could be read immediately and refrigerated for several days and still tested reproducibly to within 0.04% fat. In practice, 5-ml. portions of milk were diluted with 20 ml. of solution, both delivered by syringe for higher volumetric accuracy than pipetting.

Light wave length. Elimination of casein micelle turbidity by the use of a chelating agent permitted use of light through the visible region of the spectrum rather than just the near infrared. Accordingly, it was found that at a wave

[3] Polyoxyethylenesorbitan monolaurate, Atlas Powder Company, Wilmington, Delaware.

length of 0.6 μ, the greatest measuring sensitivity was obtained for distinguishing samples according to both homogenization pressure and fat content.

CALIBRATION CURVES AND NOMOGRAM

Preparation and measurement of standardized samples. The procedure to obtain the calibration curves from which a nomogram could be drawn consisted of taking a quantity of milk standardized at 5.00% fat by multiple Roese-Gottlieb determination. Weighed portions of this were accurately diluted with amounts of skimmilk of known fat content to give seven fractions with a range of fat down to 2.00% in steps of 0.50% fat. Each of the seven fractions yielded five samples by homogenizing at pressures of 1,600 to 3,200 p.s.i. in increments of 400 p.s.i. Each of the 35 resulting known samples was diluted with 4 vol. of the Versene solution described above, prewarmed to 40° C., and measured for the P and T readings, using a 500-μ sample light path and a wave length of 0.6 μ (orange color). The milk glass standard used as reference was one described as transmitting 50% at 1-μ wave length. More specifically, this standard transmitted 35% of the incident light of 0.6 μ, with the photocell in the close position and 9% with the photocell at a distance of 12 cm. as measured against the microcell with a water light path of 500 μ.

Calibration graph. The P and T optical density readings of the 35 samples are shown graphically in Figure 7. Two families of intersecting curves were obtained from the data, one relative to fat content and the other relative to average particle size in terms of homogenizing pressure. This picture confirmed the theory, in that the fat curves are almost truly equidistant and parallel, and the family of curves relative to particle size are very nearly linear with slopes which vary according to size [see Equations (11) and (12)]. Deviation of both families of curves from the ideal was more evident at the upper end of the fat range, where multiple scattering begins to influence the measurements. The units of both abscissa and ordinate are shown as positive when the density reading of a sample was more dense than the reference standard, and negative when the sample was less dense. In practice, the density of the sample in the former case was measured relative to the standard and in the latter case the density of the standard was read relative to the sample.

Construction of nomogram. Having two families of straight lines relative to particle size and concentration, the construction of a nomogram is very simple. A straight line plotted in a Cartesian coordinate system is depicted as a point in a nomogram where the two axes are parallel. Each family of straight lines is depicted as a series of points which can be connected to form a curve in a nomogram. Furthermore, if a family of straight lines has a common intersection point (or are parallel), then the resulting nomogram curve will also be a straight line. Once a pair of parallel axes was chosen as the boundaries of a nomogram, the points representing fat content and homogenizing pressure were found. The resulting nomogram from which unknown samples could be determined was drawn as shown in Figure 8. Further subdivisions, especially of the fat scale, were drawn according to the observed sequence of the seven subdivisions of fat from 2 to 5%.

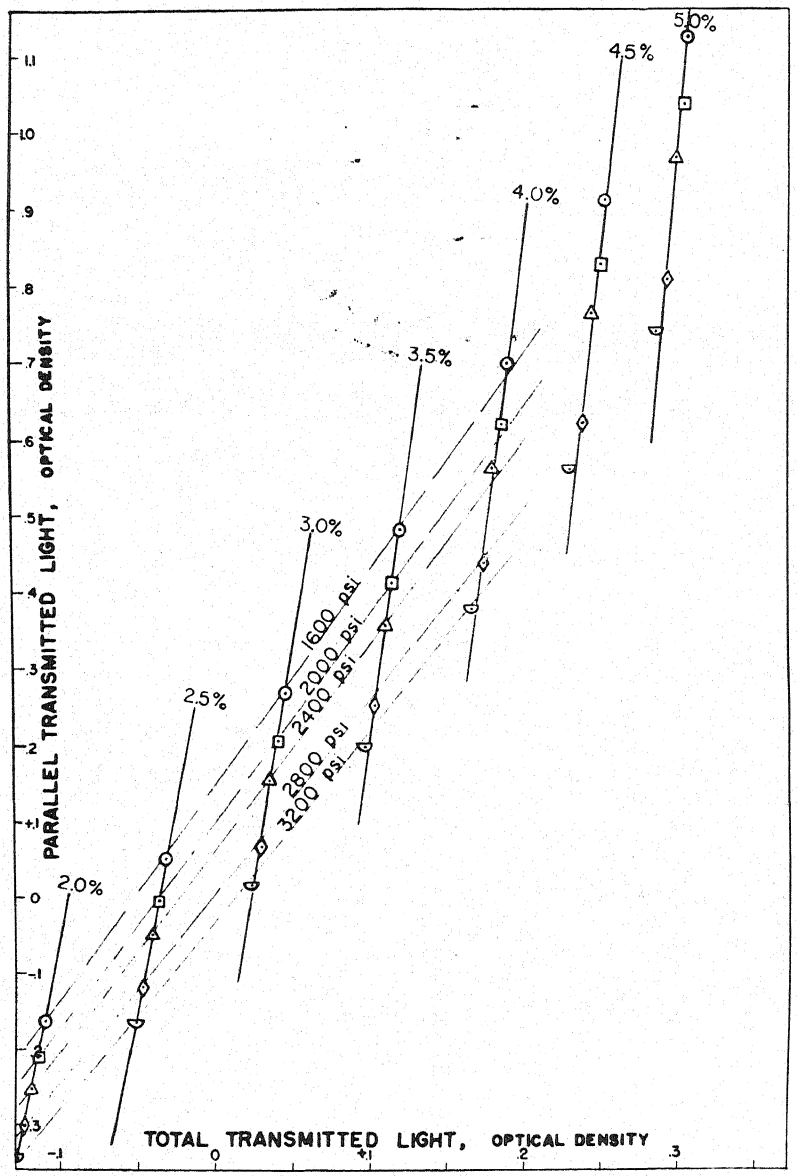


FIG. 7. Calibration curves.

To read the nomogram, the P and T readings of an unknown milk were found on the corresponding axes of the nomogram. A straight-edge was held on the two points so that a connecting line was formed between the two. The fat content and homogenization pressure were then read at those points where the connecting line crossed the respective scales.

Homogenization pressure as shown in the nomogram is an arbitrary reference scale and correlates the calibration and efficiency of the bench homogenizer. A

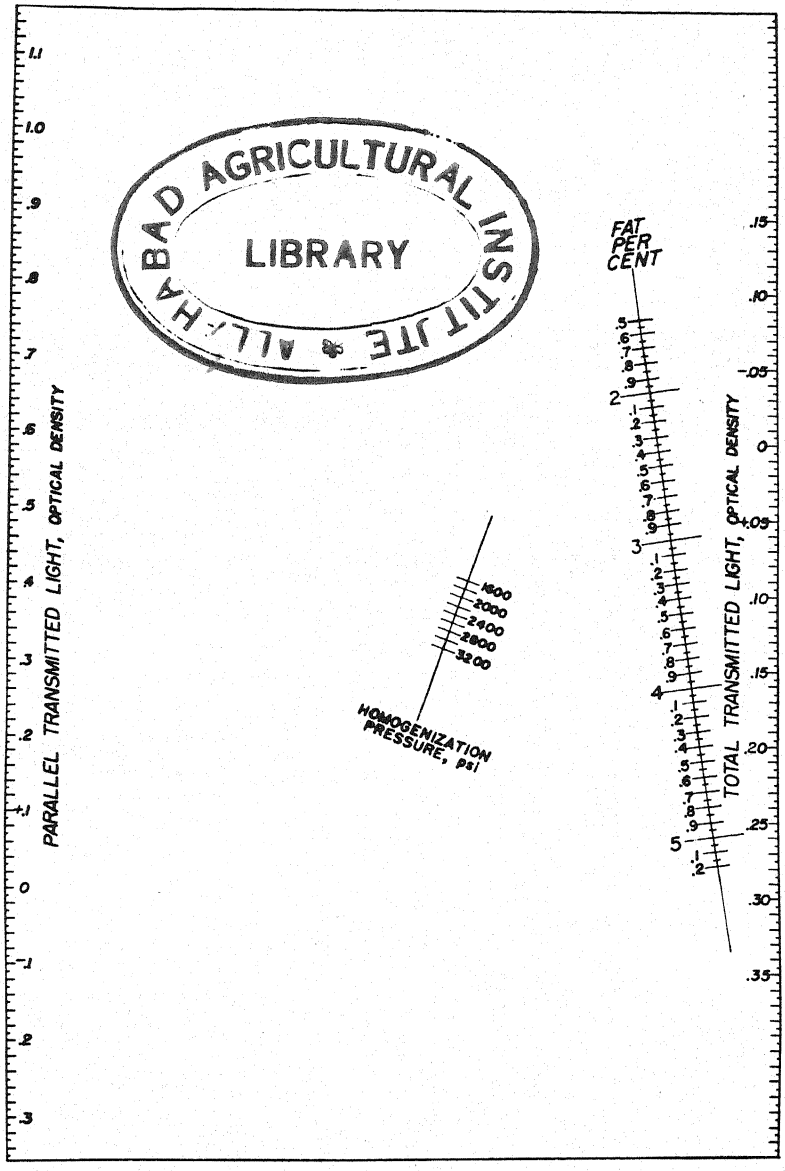


FIG. 8. Nomogram for photometric milk fat test.

scale that expressed homogenization degree in absolute units would be independent of any particular homogenizer. It is planned for the future to obtain such a scale from readings of milk fat emulsion samples fractionated according to uniform globule size.

Precision of the method. Since the 35 samples that were prepared to obtain the calibration graph and nomogram all had their fat content based on the original 5.00% fat quantity of milk, then any error in the Roese-Gottlieb fat

determination of that milk was carried through the entire fat range of the nomogram. Assuming there was no Roese-Gottlieb error on the original milk and that therefore all the prepared samples were correct, there remained only the photometric reading errors and those due to the construction and reading of the nomogram. Using the P and T optical density readings of the original 35 samples, the nomogram fat for each sample was read and compared to the original calculated fat content. From the differences between the two sets of values, the expected error of a single determination was found to be $\pm$ .05% fat, which represents the precision of the method.

COMMERCIAL MILK SAMPLES

A practical demonstration of this method consisted of reading fat content and homogenization pressures of commercial homogenized milk. The data obtained and comparative tests by the Babcock method were compiled in Table 1. The compilation includes data obtained for: 1) 45 samples of 19 different brands of milk purchased and tested through a 1-mo. period of time, 2) samples (No.25-29) mixed according to dissimilar homogenization pressure (as read on the nomogram), 3) mixtures (No. 40-43) with nonhomogenized milk, 4) samples (No. 56-67) rehomogenized at 1,600 p.s.i., 5) a reconstituted whole milk powder sample (No. 55), and 6) dilutions with skimmilk (No. 68-81).

TABLE 1
Comparative analyses of homogenized milk (continued)

Sample No.	Brand or mixture	Nomogram reading: Homogenization pressure	Nomogram reading: Fat	Babcock fat	Nomo. minus Bab.
		(p.s.i.)	*(%)*	*(%)*	
1	O	3,900	3.31	3.47	−.16
2	F	4,200	3.37	3.32	+.05
3	E	4,700	3.48	3.46	+.02
4	K	3,700	3.37	3.39	−.02
5	S	3,900	3.36	3.31	+.05
6	G	4,200	3.21	3.25	−.04
7	Q	2,800	3.43	3.45	−.02
8	A	3,200	3.52	3.54	−.02
9	J	3,000	3.38	3.31	+.07
10	B	3,200	3.74	3.58	+.16
11	B	3,000	3.43	3.36	+.07
12	N	3,600	3.46	3.42	+.04
13	C	3,000	3.49	3.62	−.13
14	M	3,700	3.52	3.52	.00
15	F	4,300	3.29	3.23	+.06
16	E	4,400	3.64	3.59	+.05
17	Q	3,000	3.43	3.47	−.04
18	S	3,800	3.14	3.06	+.08
19	H	4,300	3.30	3.31	−.01
20	K	3,800	3.52	3.53	−.01
21	A	3,600	3.50	3.43	+.07
22	I	3,600	3.40	3.41	−.01
23	O	4,200	3.17	3.17	−.00
24	D	3,600	3.21	3.37	−.16
25	No. 13 & 15, 1:1	3,600	3.51	3.42	+.09
26	No. 16 & 17, 1:1	3,700	3.63	3.53	+.10

TABLE 1
Comparative analyses of homogenized milk (concluded)

Sample No.	Brand or mixture	Nomogram reading		Babcock fat	Nomo. minus Bab.
		Homogenization pressure	Fat		
		(p.s.i.)	*(%)*	*(%)*	
27	No. 17 & 19, 1:1	3,700	3.42	3.39	+.03
28	No. 22 & 23, 1:1	4,000	3.33	3.29	+.04
29	No. 21 & 24, 1:1	3,700	3.43	3.40	+.03
30	H	3,400	3.26	3.29	−.03
31	C	3,900	3.27	3.39	−.12
32	D	3,800	3.42	3.43	−.01
33	N	2,800	3.41	3.39	+.02
34	B	3,100	3.69	3.52	+.17
35	P	3,800	3.44	3.41	+.03
36	Q	3,500	3.48	3.53	−.05
37	E	4,300	3.52	3.41	+.11
38	L	3,100	3.27	3.21	+.06
39	B	3,500	3.53	3.49	+.04
40[a]	No. 39, 9:1	4,800	3.53	3.49	+.04
41[a]	No. 39, 8:2	4,600	3.54	3.49	+.05
42[a]	No. 39, 7:3	4,400	3.53	3.50	+.03
43[a]	No. 39, 6:4	4,000	3.51	3.50	+.01
44	S	3,000	3.36	3.34	+.02
45	B	3,200	3.80	3.66	+.14
46	P	3,600	3.49	3.47	+.02
47	A	2,800	3.56	3.50	+.06
48	C	4,000	3.21	3.29	−.08
49	D	4,000	3.33	3.42	−.09
50	A	3,700	3.46	3.36	+.10
51	P	3.800	3.42	3.43	−.01
52	E	2,900	3.42	3.40	+.02
53	R	3,600	3.46	3.47	−.01
54	Q	3,100	3.44	3.57	−.13
55	W.M. powd.	4,600	3.17	3.22	−.05
56	No. 44, rehom.	3,100	3.31	3.34	−.03
57	No. 45, rehom.	3,300	3.74	3.66	+.08
58	No. 46, rehom.	3,700	3.46	3.47	−.01
59	No. 47, rehom.	2,900	3.51	3.50	+.01
60	No. 48, rehom.	4,100	3.19	3.29	−.10
61	No. 49, rehom.	4,100	3.32	3.42	−.10
62	No. 50, rehom.	3,700	3.43	3.36	+.07
63	No. 51, rehom.	3,900	3.40	3.43	−.03
64	No. 52, rehom.	3,100	3.39	3.40	−.01
65	No. 53, rehom.	3,700	3.45	3.47	−.02
66	No. 54, rehom.	3,100	3.44	3.57	−.13
67	No. 55, rehom.	4,300	3.21	3.22	−.01
68	No. 44 + skim, 9:1	2,700	2.97	3.02	−.05
69	No. 46 + skim, 9:1	3,600	3.14	3.13	+.01
70	No. 47 + skim, 9:1	2,800	3.24	3.16	+.08
71	No. 48 + skim, 9:1	3,900	2.99	2.97	+.02
72	No. 49 + skim, 9:1	3,900	3.00	3.09	−.09
73	No. 50 + skim, 4:1	3,600	2.87	2.71	+.16
74	No. 51 + skim, 4:1	2,200	2.78	2.76	+.02
75	No. 52 + skim, 9:1	3,000	3.05	3.07	−.02
76	No. 52 + skim, 4:1	3,000	2.84	2.74	+.10
77	No. 53 + skim, 9:1	3,600	3.14	3.13	+.01
78	No. 53 + skim, 4:1	3,700	2.89	2.80	+.09
79	No. 54 + skim, 9:1	3,100	3.13	3.22	−.09
80	No. 54 + skim, 4:1	3,000	2.87	2.88	−.01
81	No. 55 + skim, 9:1	3,900	2.74	2.91	−.17

[a] Mixtures with nonhomogenized milk and mixture then homogenized at 2,000 p.s.i.

Determinations of fat in mixtures of samples, mixtures with nonhomogenized milk, and rehomogenized samples were done to demonstrate the freedom of the test from a globule size distribution factor, although the absence of a systematic error according to brand was the best demonstration. Those samples diluted with quantities of skimmilk were prepared, to extend the range of fat content tested. The bench homogenizer was not used to further process commercial homogenized milk samples, except as indicated in the table of data. Readings higher than 3,200 p.s.i. were extrapolated on the nomogram scale. The test procedure on a homogenized milk sample was simply to prewarm, dilute, read P and T, align the P and T readings on the nomogram axes, and read the two scales, a procedure which took only several minutes' time. The added step of collecting in a vial a representative 15-ml. sample of milk obtained by three strokes of the bench homogenizer level adds only a minute or so to the procedure for nonhomogenized milk.

The homogenization figures reported in Table 1 demonstrate the usefulness of the additional information obtained by this method. For example, of the four tests of Brand E milk, three show a homogenization pressure reading of 4,300 to 4,700 p.s.i.; the reading of one sample was considerably lower, showing 2,900 p.s.i. This may reflect on the condition of the specific homogenizer on that particular day. The data for Brand Q, on the other hand, show a consistently lower homogenization pressure. Samples No. 25 to 28 are equal mixtures of samples which had dissimilar homogenization readings. The homogenization readings of the mixtures were, in all cases, almost the exact numerical average of the original samples. Sample No. 29 is an equal mixture of two different brands which had the same homogenization reading, 3,600 p.s.i., and the reading for the mixture was 3,700 p.s.i.

The Babcock comparative tests were run by a highly experienced analyst and read to the second decimal place. This does not represent the true accuracy of the method, which is generally assumed to be around 0.1%, but repeated analysis of coded samples has shown that it is possible for an individual analyst to consistently obtain a higher relative precision than 0.1%. As the majority of our Babcock analyses have been carried out by the same operator, we feel that it is justified to report the Babcock values with two significant decimal places. The photometric-minus-Babcock figures shown in the last column of Table 1 can be summarized as follows:

Difference of .01% fat or less: 17 samples, or, 21%
Difference of .05% fat or less: 49 samples, or, 60%
Difference of .10% fat or less: 70 samples, or, 86%
Difference of .15% fat or less: 76 samples, or, 94%
Difference of .17% fat or less: 81 samples, or, 100%

DISCUSSION

Theory. The factors concerned with a turbidimetric procedure for the milk emulsion and the mathematical development based on two independent measure-

ments were treated and applied practically to homogenized milk. Naturally, we have also used the method on nonhomogenized milk, but we did not succeed as well. This is undoubtedly because the fat globules of nonhomogenized milk fall on an unfavorable part of the a curve for light up to 1-μ wave length. Light of 3- to 4-μ wave length might possibly give equally good measuring conditions, as have been developed for homogenized milk, although it may be that the natural globule size range is too extended to be fully accommodated on the first linear section of the a curve. For this reason, homogenization of samples prior to measurement may still be required. In our studies with nonhomogenized milk from different breeds, the photometric readings could be correlated to some extent with their respective average globule size. The points representing the individual breeds lined up similarly to the way the homogenization pressures do (as shown in Figure 7).

The consequences and usefulness of a procedure of measuring two quantities were never fully exploited before, although the concept is not entirely new, as shown by Barnes and La Mer (2), Barnes and Stock (3), Sinclair (26), Powell (22), Johnson, Chiantella, and Carhart (15), Loeschke and Wever (19), and Wright (30).

Factors affecting accuracy. The main factors concerned with the accuracy and precision of the method have been discussed in detail. A few other factors which can cause variations are set forth briefly here. The relative refractive index of a scattering system being an important parameter in the theory of light scattering, gross variations in refractive index of either the fat or serum would cause erroneous fat determination by the photometric method. Variation of solids-not-fat content mainly causes the variation of serum refractive index. In milks from individual cows, the greatest variation of solids-not-fat has been reported as 7.50–10.65%, although the extremes for 3.5% fat milk are given as 8.20–8.90% (28). This is a variation of 0.70% solids-not-fat, which corresponds to a refractive index variation of 1.3449–1.3460. However, this variation is compensated to a great extent by the process of diluting milk samples before measuring photometrically, whereby the variation of solids-not-fat is 0.14% and the corresponding refractive index was found to vary from 1.3358 to 1.3361, a variation of little significance.

A similar compensating factor does not exist for milk fat, whose refractive index range is given by Rogers (24) as 1.4527–1.4566 and by Richmond (23) as 1.4524–1.4565 (converted from butryro-refractometer readings), or a difference of 0.004 unit. The seasonal variation given by the latter reference amounts to 0.0017 unit. We were not able to obtain samples of milk with fat of various refractive indexes. However, as the optical effect depends on the value of the refractive index ratio of fat and serum, it was possible to simulate any variation of the fat refractive index by a corresponding variation in the aqueous phase. Results from experiments where glucose was added to milk samples showed that a difference of 0.004 unit of refractive index caused the nomogram reading to differ by 0.12% fat. Consequently, for a nomogram constructed from milk of average fat refractive index an error of only 0.06% fat can be expected when

the fat refractive index is at either limit of the range. Milk from individual cows would occasionally be more liable to be found at either limit than would herd milk.

Natural variations of milk pH, between 6.2 and 6.7, did not affect the fat determination by virtue of using a diluting solution of pH 10 which maintained pH of dilutions in the range of 8 to 9.

Several other factors which have not been investigated fully are the effect of clarifier slime content in raw milk and the effect of the commonly used preservatives. In reference to the latter topic, it was noticed that the concentration of Versene in the diluting solution is more than sufficient to have a bacteriostatic effect.

Application to other dairy products. The measuring procedure as described for milk appeared to be applicable to any dairy product but, from a practical point of view, the upper limit of fat content that can be determined is around 40%. For higher fat contents, the amount of dilution necessary becomes correspondingly higher and, as a result, the experimental error increases in proportion to the dilution factor. To satisfy the condition that there be neither coherent nor multiple scattering by the fat emulsion, the actual fat concentration of the diluted sample should not exceed 1%.

Products which are not fluid to begin with can be reconstituted by neutralizing and redissolving the protein curd. Products with fat content lower than that of whole milk require less dilution. The method of determining fat photometrically has been extended to various products such as cream cheese, ice cream mix, heavy cream, sour cream, evaporated milk, creamed cottage cheese, yoghurt, buttermilk, and skimmilk. The results with these products will be reported in a future publication.

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A METHOD FOR THE DETERMINATION OF RADIONUCLIDES IN MILK ASH

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SUMMARY

A modified method suitable for routine monitoring for the determination of four radionuclides [total radiostrontium, strontium^{90} (yttrium^{90}), barium^{140}, and cesium^{137}] in the ash from one liter of milk, is described. With this method, based on radiometric analyses and employing β- or γ-counting as applicable, recoveries are as follows: strontium, $82 \pm 2\%$; yttrium^{90} (strontium^{90}), $77\% \pm 4\%$; barium^{140}, $85\% \pm 4\%$; and cesium^{137}, $96\% \pm 3\%$. The over-all recovery of strontium^{90} (yttrium^{90}), taking into account the recovery of strontium, is $64\% \pm 5\%$. These values indicate sufficient precision for most purposes. The major reason for loss of strontium and barium activity in the nitric acid treatment step is the low acid concentration (64–65%) employed. The interference of strontium on barium and barium on strontium is less than 1%, whereas the presence of cesium has no effect on the determination of strontium and barium activity. The results obtained on milk samples using this method are presented elsewhere (6).

A number of laboratories are currently determining the concentration of various radionuclides in milk. Among the procedures in use are detailed chemical separation (2, 5, 10, 12, 13), ion-exchange (11), and γ-ray spectroscopy (1, 3). Determination of cesium and alkaline earth radionuclides is complicated by some of the natural milk ash components, particularly calcium, potassium, and phosphate. Radiochemical methods for the separation of these radionuclides must take cognizance of the presence of these interfering materials.

Bergh *et al.* (2), Bryant *et al.* (5), Harley (10), Martell (12), and Merten (13) separate strontium and barium from milk ash as nitrates by treatment with concentrated HNO_3. They remove rare earths as hydroxides, separate barium as chromate at pH 5.0, and determine strontium^{90} on the basis of the activity of its daughter yttrium^{90}, which is precipitated as the oxalate after an ingrowth period. Volchok *et al.* (17) precipitate alkaline and rare earths from milk ash as oxalates and remove the rare earths as hydroxides. After ingrowth, the yttrium and lanthanum are separated from the alkaline earths and used for the determination of strontium and barium, respectively.

Cesium is commonly separated from potassium as a silicotungstate (5) or as alkali alum (10) and, finally, is precipitated as chloroplatinate.

Since our program included determinations of strontium^{89}, strontium^{90}, barium^{140}, cesium^{137}, and total rare earths + Zr^{95} – Nb^{95} in mlik on a routine monitoring basis, it was deemed necessary to modify available procedures so that: (a) all the radionuclides of interest could be obtained on a single sample of milk ash from one liter of fluid milk; (b) the radionuclides would be recovered in high yield with such consistency that chemical yield determinations would not be necessary on each sample; (c) the final counting samples would be suitable for use on

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the high-efficiency counting instrumentation[1] at the Center; and (d) the methods could be successfully carried out by technicians in routine analyses.

The chemical analysis of the initial milk samples disclosed no significant concentrations of either the rare earths or $Zr^{95} - Nb^{95}$; therefore, the procedure was simplified to eliminate these materials. The techniques which serve as the basis for the final procedure are: (a) removing phosphate (and calcium) by nitric acid treatment, (b) determining strontium^{90} by yttrium^{90} extraction (7), and (c) recovering cesium from nitric-acid supernatant by precipitation as phosphomolybdate (11), with further purification by solution of the phosphomolybdate and reprecipitation of the cesium with cobaltinitrite (8).

EXPERIMENTAL PROCEDURE

A. Reagents

All chemicals and solvents employed are reagent grade, unless otherwise stated.

1. Acetic acid—glacial.
2. Ammonium acetate buffer (pH 5.0). Prepare by mixing one part of 1.5 *N* acetic acid with two parts of 3 *N* ammonium acetate.
3. Ammonium acetate wash solution. Dilute three parts of ammonium acetate buffer with 20 parts of water.
4. Ammonium hydroxide—6 *N*.
5. Ammonium molybdate. Dissolve 100 g. of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in water and make up to one liter.
6. Barium nitrate—1 *N*.
7. Cerium + zirconium carrier. Equal volumes of 0.1 *N*. $Ce(NO_3)_3 + 0.1\ N\ ZrOCl_2 +$ HCl necessary to prevent hydrolysis.
8. Ferric chloride—1 *N*.
9. *n*-Hexane.
10. Hydrochloric acid—6 *N*.
11. Nitric acid—16 *N*.
12. Nitric acid—10% (v/v).
13. Nitric acid—1 *N*.
14. Methyl orange indicator—0.1% (w/v) in water.
15. Mixed indicator—0.1% (w/v) thymol blue + 0.1% (w/v) phenolyphthalein, in 50% alcohol.
16. Phosphoric acid—85%.
17. Sodium carbonate—3 *N*.
18. Sodium cobaltinitrite reagent. Dissolve 23 g. of $NaNO_2$ in 50 ml. water. Add 7 g. of glacial acetic acid and 2.6 g. of $Co(CH_3COO)_2 \cdot 4\,H_2O$ to the solution and let stand overnight. Filter and dilute to 100 ml. with water. Store in cold. (*Note:* Even in cold, this solution does not keep indefinitely. It should be made up in small quantities.)
19. Sodium hydroxide—6 *N*.

[1] Model PCC-11A, Shielded Internal Proportional Counter, Nuclear Measurements Corp., Indianapolis, Indiana. Model CE-14, Low Background Beta Counter, Tracerlab Inc., Richmond, California.

20. Sodium chromate—1 *N*.
21. Strontium, barium, and cesium carrier. Dissolve 211.6 g. of $Sr(NO_3)_2$, 26.1 g. of $Ba(NO_3)_2$, and 19.5 g. of $CsNO_3$ in water and make up to 1 liter.
22. Sodium nitrate—1 *N*.
23. Sodium nitrite—solid.
24. TTA solution—5% (w/v). Dissolve 5 g. of 2-thenoyl-trifluoroacetone in monochlorobenzene and make up to 100 ml.

B. Preliminary sample preparation

1. To one liter of whole milk at 20° C., add 1 ml. of mixed Sr, Ba, and Cs carriers and partially ash in a "Vycor" silica dish (15).
2. Complete the ashing in an electric muffle furnace at 450° C. for 18 hr.

C. Chemical separation of radionuclides

1. Treatment with nitric acid:
   a. Transfer ash to a 250-ml. beaker. Dissolve the residual ash in the ashing dish with 5–10 ml. of 6 *N* HCl by warming. Transfer the solution to the beaker.
   b. Add 10 ml. of 6 *N* HCl to the ash in the beaker, and heat to near dryness.
   c. Add 10 ml. of conc. HNO_3 and heat to near dryness.
   d. Add 20 ml. of conc. HNO_3 and heat to near dryness.
   e. Add 40–50 ml. conc. HNO_3 and evaporate to about 30 ml. (Note 1).
   f. Transfer into a 50-ml. glass centrifuge tube, using a little concentrated HNO_3 to facilitate transfer. Cool in an ice bath for 15 min.; centrifuge. Decant supernatant, retaining it for Part V.
   g. Add 3 ml. water (Note 2) and 10 ml. concentrated HNO_3 to the residue (Note 3); warm in a water bath at 80° C. to promote solution. Add another 20 ml. of conc. HNO_3 and continue heating for another 10 min. with occasional stirring. Cool in an ice bath for 15 min.; centrifuge. Transfer supernatant and combine it with supernatant (Step f) retained for Part V.
   h. Wash the solid (Note 4), if any, in the beaker (Step e) into the centrifuge tube with about 10 ml. of water. Warm the solution and make it alkaline (pH 9.0) with 6 *N* NH_4OH, using 3 to 4 drops of mixed indicator. If no precipitate forms, omit Steps i and j (Note 5).
   i. Centrifuge and decant supernatant to a 150-ml. beaker, retaining it for Step k. Add a little water and 10–20 ml. of 3 *N* Na_2CO_3 to residue. Digest in a water bath at 80° C. for 30 to 45 min. with occasional stirring; centrifuge. Decant supernatant, combining it *(Caution)* with the supernatant retained for Part V.

Note 1. It will be helpful to make a rough calibration of the beaker beforehand.
Note 2. All the volumes in Step g must be measured accurately to keep the concentration of HNO_3 at 64 to 65%.
Note 3. The residue is largely strontium and barium nitrates.

j. Dissolve the precipitate in 1–2 ml. of 6 *N* HCl and again test for phosphate with 6 *N* NH_4OH as before. If a large precipitate forms, repeat Steps h and i. If not (Note 6), combine supernatant with supernatant retained for Step k.

k. Add excess (5 ml.) 3 *N* Na_2CO_3 to the combined solutions (Note 7) with stirring. Allow the mixture to stand for 5 min. with occasional stirring. Centrifuge; discard supernatant after testing it for completeness of precipitation with Na_2CO_3.

2. Separation of strontium and barium

a. Dissolve the precipitate (Step 1-k) in about 2 ml. of 6 *N* HCl; warm to promote solution and to remove any CO_2. Neutralize the solution with 6 *N* NH_4OH, using methyl orange indicator. Dilute to 20 to 25 ml. with water. Add 10 ml. of ammonium acetate buffer. Warm solution in water bath, add 1 ml. of 1 *N* Na_2CrO_4, and digest in water bath for 5 min. Add 1–2 drops of 1 *N* $Ba(NO_3)_2$ and continue digesting for another minute or two with occasional stirring.

b. Add 5 ml. of water to $BaCrO_4$ precipitate, warm a little for 1–2 min., cool, centrifuge, and discard the washing.

c. Transfer the $BaCrO_4$ to a tared planchet,[2] dry at 70 to 80° C.; cool in desiccator and weigh. Determine β-activity (Note 8) in an internal proportional counter (9).

d. Make supernatant alkaline with 6 *N* NH_4OH and precipitate strontium with 3 to 5 ml. of 3 *N* Na_2CO_3. Centrifuge and discard the supernatant after testing it for completeness of precipitation with Na_2CO_3.

e. Wash the precipitate once with 25 ml. of water containing a drop of 3 *N* Na_2CO_3. Centrifuge and discard the washing.

f. Dissolve the precipitate in about 2 to 3 ml. of 6 *N* HCl, warm, dilute to 20 to 25 ml. with water. Add 1 to 2 drops of 1 *N* $FeCl_3$, heat in water bath, and make alkaline with 6 *N* NH_4OH. Digest for 5 min. Add an additional 1 to 2 drops of 1 *N* $FeCl_3$ and digest for 1 or 2 min. Remove the precipitate by filtration and discard.

g. Add to the filtrate 1 to 2 drops of Ce + Zr carrier solution, dissolve any precipitate that forms with 6 *N* HCl. Warm solution and make alkaline with 6 *N* NH_4OH. Record time (Note 9). Remove precipitate by filtration and discard it.

[2] Aluminum milk bottle caps are satisfactory for this purpose.

Note 4. The beaker may still contain a little untransferred precipitate.
Note 5. A precipitate at this point may indicate unremoved phosphate.
Note 6. A small precipitate of silica which may be formed is discarded.
Note 7. These solutions contain strontium and barium.
Note 8. α-activity may also be determined as a rough measure of Ra^{226} content.
Note 9. This is the time from which the ingrowth of Y^{90} is calculated.

h. Add to the filtrate about 5 ml. of 3 *N* Na_2CO_3 to precipitate the strontium. Centrifuge and discard supernatant after testing it for completeness of precipitation. Wash precipitate with water containing a drop of 3 *N* Na_2CO_3 and discard washing (Note 10). Set aside precipitate for 2 wk. for ingrowth of Y^{90} (Note 11).

3. Extraction of yttrium^{90}

a. Dissolve the $SrCO_3$ (Step C-2-h) in 2 to 3 ml. of 6 *N* HCl and transfer quantitatively to a 60-ml. separatory funnel with 20 to 25 ml. water. Neutralize with 6 *N* NH_4OH, using methyl orange indicator. Add 10 ml. of ammonium acetate buffer. Extract the Y^{90} from solution thrice, shaking each time for 5 min. with 5-ml. portions of TTA solution. Collect organic layers in a fresh separatory funnel. Save aqueous layer for total strontium determination.

b. Add 20 ml. of *n*-hexane to the organic layer, to make it lighter than the aqueous phase. Wash solution twice with 5-ml. portions of acetate wash solution, combine washings with aqueous phase saved for total Sr. Back extract Y^{90} from organic phase three times with 5-ml. portions of 1 *N* HNO_3, transfer HNO_3 extract to a stainless steel planchet, evaporate to dryness. Flame planchet to remove residual acid or organic matter, cool, and determine Y^{90} activity in a low background counter (4). Repeat counting three days and five days after extraction to confirm purity of Y^{90} by its rate of decay.

c. Note time and date of counting.

4. Total strontium

Heat the aqueous phase (C-3-b) on a hot plate to remove any residual organic solvent. Make solution alkaline (pH 9.0) with 6 *N* NH_4OH and precipitate strontium with 3 *N* Na_2CO_3 as before. Wash precipitate, transfer it to a tared aluminum planchet, dry, cool, and weigh. Determine β-activity as before.

5. Determination of cesium activity

a. Evaporate supernatant from Step C-1-f to dryness.

b. Dissolve residue in about 100 ml. of 10% HNO_3; add 2 ml. of 85% H_3PO_4.

c. Warm solution to 50° C., add 40 ml. of ammonium molybdate solution, and stir solution mechanically for at least half an hour.

d. Remove cesium ammonium phosphomolybdate precipitate by filtration through a sintered glass (fine porosity) crucible. Wash precipitate thrice with 50-ml. portions of 1 *N* $NaNO_3$.

Note 10. Total Sr activity may be determined, if desired, at this time.

Note 11. After 2 wk. ingrowth, the activity of Y^{90} is 97% of that of the Sr^{90} present.

e. Add 5 ml. of 6 *N* NaOH to the beaker to dissolve any untransferred precipitate; pour into the filter crucible. When the precipitate has dissolved, collect the solution in a 50-ml. glass centrifuge tube. Wash beaker and crucible several times with a little water and collect washings in same centrifuge tube.
f. Add 2 g. of $NaNO_2$ and 5 ml. glacial acetic acid to the solution and heat in a boiling water bath for at least 10 min. (Note 12).
g. Cool in an ice bath, add 3 ml. of sodium cobaltinitrite solution, and let stand in ice bath for 10 min. with occasional stirring.
h. Remove the precipitate by centrifugation; discard the supernatant after testing it for completeness of precipitation.
i. Transfer the precipitate to a tared aluminum planchet, dry, cool, and weigh. Determine β-activity in the sample as before.

SPECIAL PROBLEMS IN COUNTING LOW-LEVEL β-ACTIVITY

Efficiency of β-activity counter. To convert the observed counting rates of samples to absolute activity (disintegrations per minute), the efficiency of the counter must be determined. The efficiency, E, of an internal proportional counter is a function of counter geometry, G, backscattered radiation, B, and transmission of radiation through sample solids, T (self-absorption factor), written $E = GBT$. In the present study, values for G and B were obtained from Nader *et al.* (16); whereas, the values for T were experimentally evaluated. Typical curves for counting yields due to self-absorption for strontium^{90}, barium^{140}, and cesium^{137} are presented in Figures 1, 2, and 3.

In the low background anticoincidence counter which was employed for counting yttrium^{90} activity only, the efficiency was determined, using NBS standards of strontium^{90}–yttrium^{90}. The yttrium^{90} was separated from strontium^{90} according to the procedure described by Goldin (7). Aliquots of yttrium^{90} were plated on the planchets employed for milk samples and counted. The absolute counting rates of Y^{90} were checked by counting similar aliquots in a 4 π counter. After making necessary corrections for dead time and decay, efficiency of the counter was calculated, relating the observed activity to absolute activity.

Treatment of data. To convert counting rates of sample to absolute units ($\mu\mu$c.), the observed counts per minute are divided by 2.22 ER, where E is the fractional efficiency of the counter, R is the fractional chemical recovery, and 2.22 disintegrations per minute are 1 $\mu\mu$c.

Chemical recovery of the various nuclides was determined by tracer techniques, as indicated under Evaluation. For strontium, the results were checked by determining the chemical recovery of the added carrier by flame spectrophotometry and were found to be similar.

Since barium^{140} has a half-life of 12.8 days, the observed counting rates must be corrected for (a) ingrowth of lanthanum^{140} between separation of barium from strontium and time of counting, and (b) decay since collection of the sample.

Note 12. $NaNO_2$ is used to destroy ammonium ions.

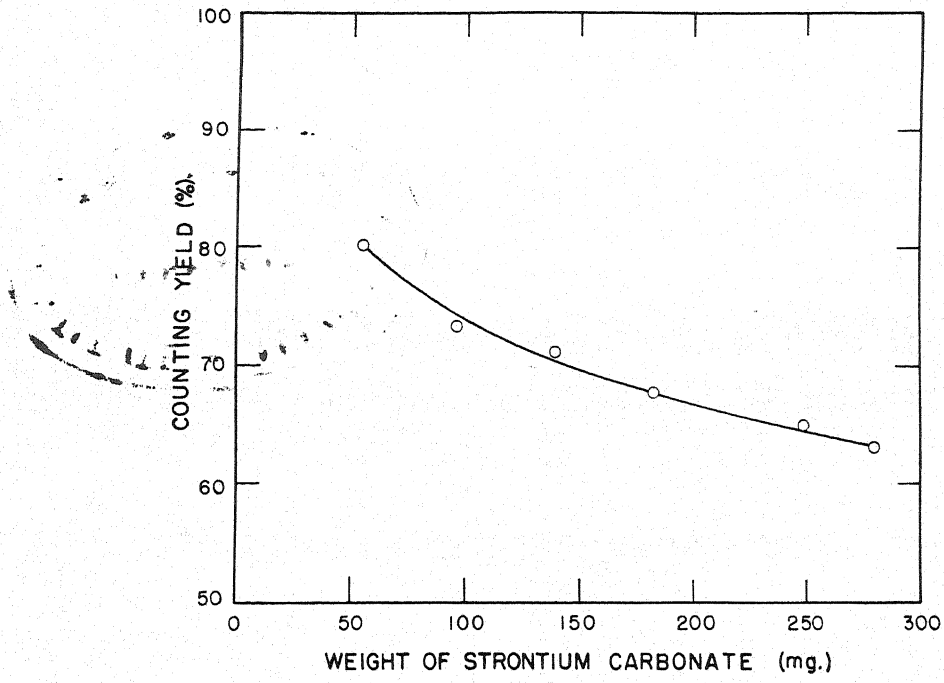


FIG. 1. Counting yield of strontium^{89} due to self-absorption.

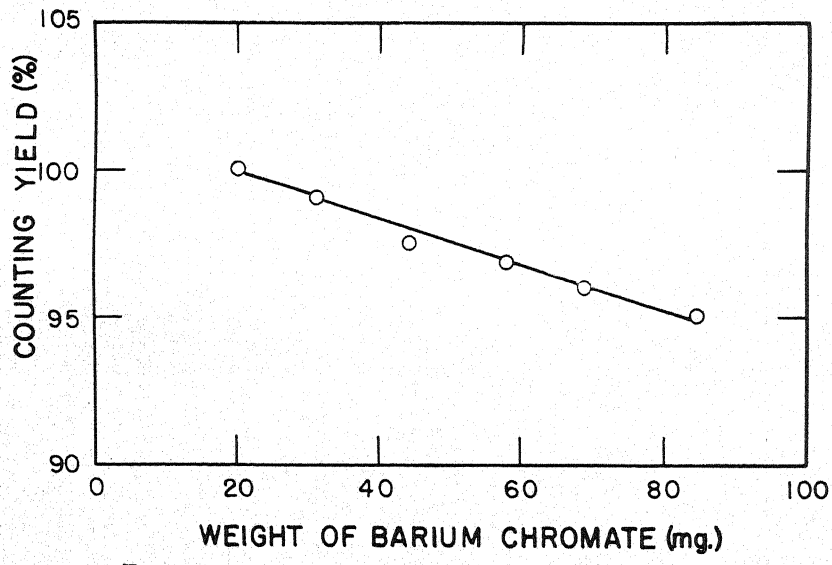
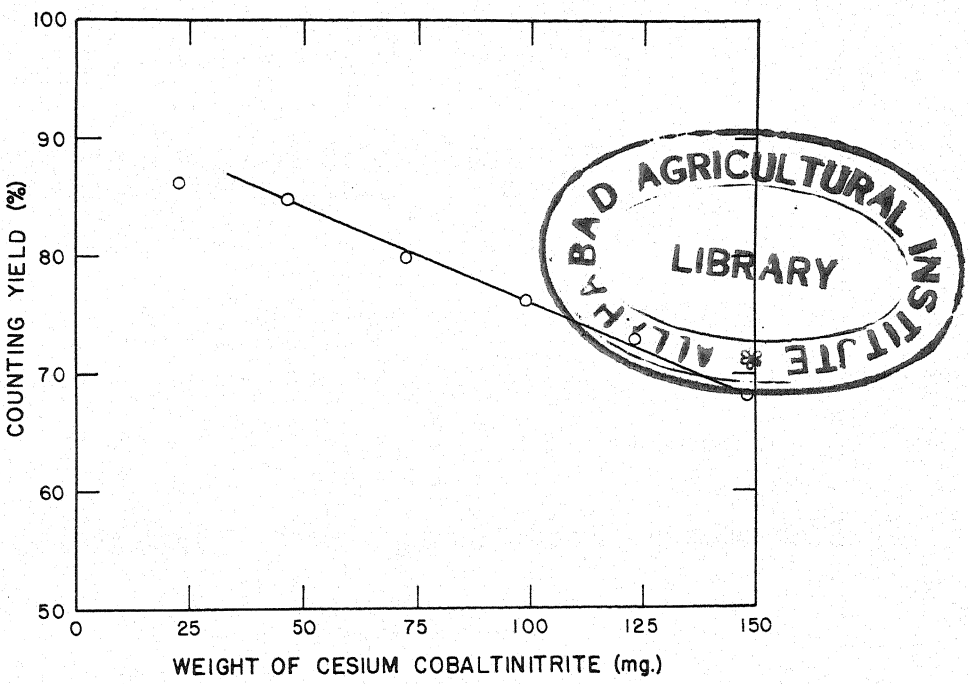


FIG. 2. Counting yield of barium^{140} due to self-absorption.



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FIG. 3. Counting yield of cesium[137] due to self-absorption.

The former factor was ignored, since the samples were counted immediately after separation and the barium [140] activity was not of sufficient consequence to warrant high accuracy. Yttrium[90] has a half-life of 64 hr., making it necessary to correct observed activity to time of separation from strontium[90] by the relationship:

$$A = A_o^{-\lambda t}$$

where A is observed activity, A_o is activity at time of separation, t is interval between time of separation of yttrium[90] and counting, and λ is the decay constant equal to $0.693/t_{1/2}$, where $t_{1/2}$ is the half-life of yttrium[90]. The corrected counting rates are converted to absolute units, as indicated above, taking into consideration the fractional chemical yields of strontium and yttrium, respectively.

EVALUATION

All milk ash samples employed in the evaluation were prepared by ashing 90 g. of nonfat dry milk solids with added strontium, barium, and cesium carriers.

In general, the method was evaluated by tracer techniques. The samples, with appropriate radionuclides, were processed as described under Procedures, the fraction or fractions of interest isolated, and recovery determined by gamma- or beta-counting as applicable. The following isotopes were employed for evaluation: $Sr^{(85+89)}$, Ba^{140} ($+La^{140}$), Cs^{137}, and Y^{91}.

Strontium. The recovery of strontium added to milk ash is 82% ± 2% (Table 1). Loss of strontium in the barium fraction was less than 1%, and in

TABLE 1

Recovery of added $Sr^{(85+89)}$ to milk ash

Sample	γ-Activity (counts/min)		Recovery
	Added	Found	
			(%)
1	480	390	81
2	960	820	85
3	1,920	1,540	80
4	2,880	2,300	80
5	3,840	3,100	81
			Av. 81±2

the nitric acid supernatant was 8% ± 2%, the latter constituting the major strontium loss.

Yttrium extraction. Yttrium is extracted from solutions buffered at pH 5.0 (7). Since the extraction of yttrium with TTA is a function of pH, it was necessary to determine whether the pH adjustment is critical.

Twenty-milliliter portions of ammonium acetate buffer solutions were adjusted to pH values of approximately 2.4, 4.4, 4.9, and 6.25. The solutions were transferred to 60-ml. separatory funnels. A 1-ml. solution of Y^{91} with known activity was added to each sample, and extracted with 5 ml. of TTA solution. The aqueous and organic phases were separated and their β-activities determined. The separatory funnel was then washed thrice with 5-ml. portions of water and the washings discarded. Each separatory funnel was washed with 4 ml. of 1 *N* HCl and the β-activity of the acid wash measured to determine the amount of Y^{91} which had been absorbed on the glass.

The results presented in Table 2 show the variation of yttrium extraction by TTA with pH. The optimum pH is between 4.4–5.0 under the experimental conditions studied. At lower pH, less yttrium is extracted, while at higher pH, there is a tendency for greater loss of Y^{91} by adsorption on the glass. Therefore, for best extraction of Y^{91}, a pH close to 5.0 is required.

Recovery of yttrium. Varying amounts of Y^{91} were added to 1-ml. portions of 2 *N* $Sr(NO_3)_2$ solution in 60-ml. separatory funnels. Strontium nitrate was added to replace $SrCO_3$ in the regular samples. The solutions were treated as described under Section C-3. The results are presented in Table 3.

TABLE 2

Effect of pH on the extraction of Y^{91} with TTA

pH	β-Activity (counts/min)					
	Added	Aqueous phase	TTA phase	Total (Aqueous + TTA)	On glass wall	Recovery
						(%)
2.4	43,160	36,000	450	36,450	30	84
4.4	43,160	8,000	33,250	41,250	120	96
5.0	43,160	2,400	38,850	41,250	80	96
6.3	43,160	200	38,200	38,400	470	89

TABLE 3
Recovery of added Y^{91}

Sample	β-Activity (counts/min)		Recovery
	Added	Found	
			(%)
1	60	48	80
2	119	82	69
3	129	144	83
4	210	161	76
5	210	167	79
6	210	161	77
7	239	183	77
			Av. 77±4

The recovery of Y^{91} was 77% ± 4%. Since the extraction of Y^{91} by TTA is almost quantitative, the low recovery is due to incomplete back-extraction of Y^{91} by 1 *N* HNO_3.[3]

Effect of yttrium carrier on recovery. Conceivably, recovery of Y^{91} might be improved by addition of yttrium carrier prior to TTA extraction. This hypothesis was tested by measuring the extraction of Y^{91} from samples with and without 0.01 meq. of yttrium carrier. No difference in the results was observed, indicating that the yttrium carrier has no effect upon the recovery of added Y^{91}.

Barium. To determine the effect of pH on the precipitation of barium chromate, 20-ml. portions of ammonium acetate buffer solutions adjusted to pH values of 1.5, 3.5, 4.0, 4.5, 5.0, and 6.5, respectively, were placed in 50-ml. centrifuge tubes. One milliliter of 0.1 *N* $Ba(NO_3)_2$ and tracer Ba^{140} (+La^{140}) were added, and barium chromate was precipitated in the usual manner. The $BaCrO_4$ precipitate was separated and allowed to stand for approximately 10–12 days so that transient equilibrium would be established between Ba^{140} and its daughter La^{140}. The γ-activity of the various samples was measured on a γ-ray spectrometer, using the Ba^{140} peak at 170 Kev. and the La^{140} peak at 830 Kev.

The data in Table 4 show that precipitation of $BaCrO_4$ is independent of pH in the range 3.5 to 6.5, falling off slightly at pH 1.5. Although pH adjustment

[3] If 1 *N* HCl is used instead of 1 *N* HNO_3, recovery of yttrium is increased to 95%. However, this necessitates use of copper planchets, which are less resistant to oxidation or heating than stainless steel.

TABLE 4
Precipitation of barium chromate at various pH's

pH	γ-Activity (counts/min)					
	Ba^{140} peak at 170 Kev		Ba pptd.	La^{140} peak at 830 Kev		Ba pptd.
	Added	Found		Added	Found	
			(%)			(%)
1.5	29,060	26,540	91	17,700	16,290	92
3.5	29,060	27,670	95	17,700	17,040	96
4.0	29,060	28,170	97	17,700	17,300	97
4.5	29,060	28,030	96	17,700	17,000	96
5.0	29,060	27,550	95	17,700	16,900	95
6.5	29,060	27,660	95	17,700	17,010	96

exactly to 5.0 is not a critical factor, as far as $BaCrO_4$ precipitation is concerned, it is necessary to control the pH at 5.0 or less to avoid strontium contamination. The results are the same whether calculated from the barium or lanthanum peak, since transient equilibrium between Ba^{140} and La^{140} was reached.

The results on the recovery of added Ba^{140} to milk ash samples are presented in Table 5. Again, the recoveries calculated from barium and lanthanum peaks are the same. On an average, 85% ± 4% of the added Ba^{140} was recovered. Contamination of barium in the strontium fraction was not detectable. As in strontium, the major source of barium loss was the nitrate separation step.

Cesium. The data presented in Table 6 show that approximately 96% ± 3%

TABLE 5

Recovery of Ba^{140} added to milk ash

Sample	γ-Activity (counts/min) Ba^{140} peak at 170 Kev			γ-Activity (counts/min) La^{140} peak at 830 Kev		
	Added	Found	Recovery	Added	Found	Recovery
			(%)			(%)
1	29,060	25,660	85	17,700	15,630	88
2	29,060	25,360	87	17,700	15,580	88
3	29,060	23,840	82	17,700	15,550	82
4	29,060	25,660	88	17,700	15,660	88
5	29,060	25,010	86	17,700	15,100	85
6	29,060	22,950	79	17,700	13,790	78
			Av. 85±4			Av. 85±4

TABLE 6

Recovery of added Cs^{137} to milk ash

Sample	γ-Activity (counts/min) Added	Found	Recovery
			(%)
1	9,740	9,270	95
2	9,740	9,000	92
3	9,740	9,540	98
4	9,740	9,180	94
5	9,740	9,640	99
			Av. 96±3

of the Cs^{137} added to milk ash is recovered. Although this is very satisfactory, it should be recognized that in order to obtain true cesium values, the milk should be ashed below 450° C., since above this temperature severe losses of Cs^{137} occur (18).

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A METHOD FOR THE RAPID ASHING OF MILK FOR RADIONUCLIDE ANALYSIS

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SUMMARY

A method for the rapid ashing of milk and milk products for analysis of radionuclides has been described. Based on the results of calcium analysis, this method is reproducible and accurate and is in agreement with the results obtained by the usual ashing procedures. With this method, ashing time for liter quantities of milk is shortened, and only the nominal attention of the operator is required, other than assembling and cleaning the apparatus. It has been found that with adequate equipment, one technician can reduce six to eight samples of milk to ash in one work day. This rapid method has been a major factor in establishing the practicality of a routine monitoring program for radionuclides in milk.

An initial step in most radiochemical analyses of biological substances is the removal of organic materials. This is generally accomplished by either wet or dry ashing of the sample. The several ashing procedures suitable for milk or similar products (1, 5, 7) have been designed for relatively small quantities of sample. For example, in wet ashing (5), a sample (5–100 ml.) is digested with concentrated nitric acid in a Kjeldahl flask until brown fumes cease to be evolved and it is then treated with perchloric acid until heavy white fumes appear. In dry ashing (1, 7), the sample (5–100 ml.) is evaporated to dryness in a suitable container over a steam bath or in an oven maintained at 105 to 110° C., after which it is ashed in a muffle furnace.

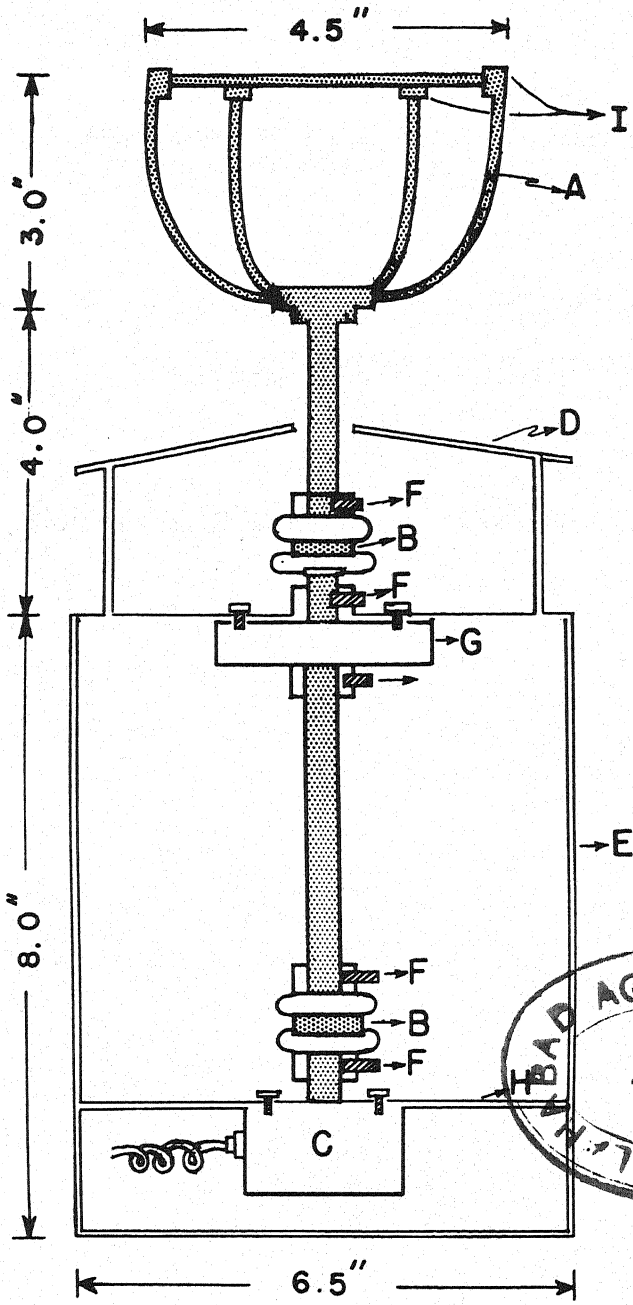
The determination of specific radionuclides in fluid milk as conducted by the Public Health Service (3) requires a large sample (one liter) because of the low concentrations of radionuclides. In a routine operation, considerable attention must necessarily be given to efficient and rapid methods of analysis. The results of preliminary trials indicated that scaling-up the conventional ashing would not be satisfactory. Both wet and dry techniques were found to be slow and to require almost constant attention from the analyst. In spite of these disadvantages, various laboratories (4, 6) have adapted them for ashing large quantities of milk. A rapid technique has been developed by the Atomic Energy of Canada (2), but it was found to be unsatisfactory for routine analysis. Therefore, to ash large quantities of milk in a short period, and to process large numbers of samples, the rapid method presented here has been developed.

MATERIALS AND METHODS

Apparatus

a. "Vycor" silica evaporating dish, 350-ml. capacity.
b. Blast burner suitable for use with compressed air and gas.
c. Low-volume variable flow pump, 0 to 20 ml. per minute.
d. Rotating evaporating dish holder. Construction details for a satisfactory

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FIG. 1. Construction details of the rotating evaporating dish holder: (A) metal frame for evaporating dish, (B) insulator coupling, (C) back gear electric motor (6 or 8 r.p.m.), (D) asbestos sheet covered with aluminum foil, (E) ventilated enclosure, (F) screws, (G) ball bearing, (H) shelf for supporting motor, (I) asbestos padding.

holder are presented in Figure 1. Although the design is not critical, the unit must be built to operate under high ambient temperatures.

e. Separatory funnel, two-liter capacity.
f. Water-cooled condenser, 200 mm. in length, with modified water inlet (Figure 2).
g. Dispensing nozzle. The dispensing nozzle consists of a glass tube, 30 to 35 mm. in length by 5 mm. inside diameter, one end of which is pulled to a tapered tip and bent at an angle of 45 to 60°.
h. Muffle furnace.
i. Hood or suitable enclosure for operation of equipment.

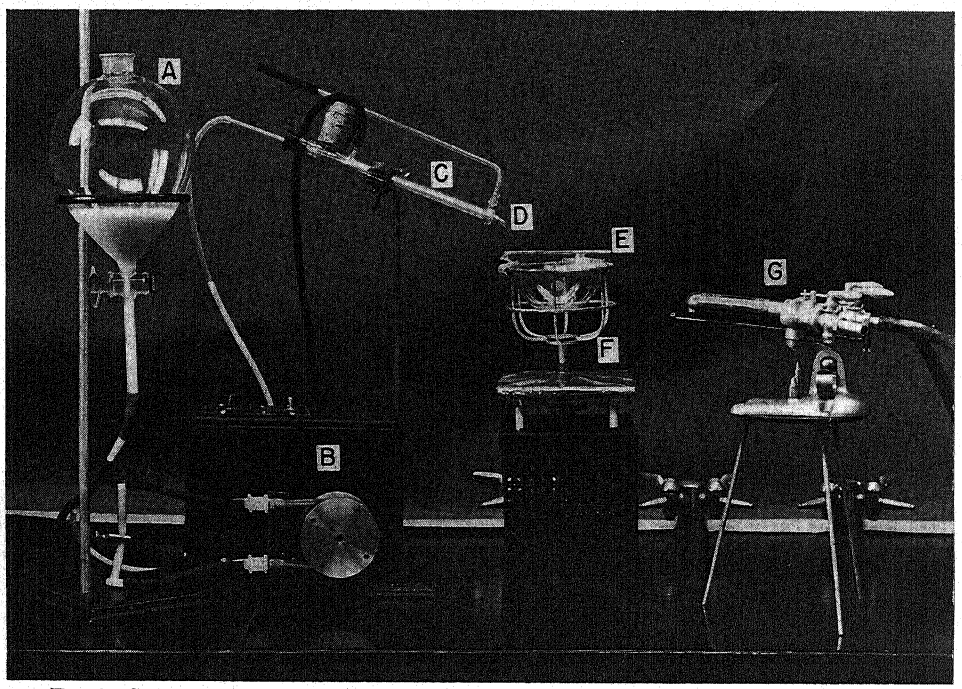


FIG. 2. Complete assembly for rapid ashing of milk: (A) separatory funnel, (B) variable flow pump, (C) water-cooled condenser, (D) dispensing nozzle, (E) "Vycor" silica dish, (F) rotating dish holder, (G) blast burner.

Procedure. One liter of milk at 20° C. is transferred from a volumetric flask to a separatory funnel. A rubber tube attached to the end of the funnel is connected to the variable flow pump. The funnel and the pump are placed outside the hood to protect them from the heat of the blast burner. The dispensing nozzle is passed through the water-cooled condenser and is connected to the pump outlet by means of a rubber tube. The condenser is placed at approximately a 90° angle to the burner to avoid overheating the nozzle. The evaporating dish in which the milk is ashed is placed in the rotating dish holder which fits into the metal ring of the turntable. The dish is heated directly by the blast burner and the intensity of the flame is controlled by adjusting the amount of gas or air. The milk is pumped

from the flask through the dispensing nozzle and is allowed to drop into the heated crucible at approximately 5 to 8 ml. per minute. The rate of flow and intensity of heat are so adjusted that the milk is completely evaporated and partially charred and no fluid milk accumulates in the evaporating dish. The condenser keeps the milk cool and prevents heat coagulation in the nozzle. Splattering is minimized by maintaining a distance of about 0.5 in. between the nozzle and the lip of the crucible. The cushioning action of the charred milk as it collects in the dish also aids in this regard.

After the liter sample has been pumped into the evaporating dish, requiring usually about 2.5 to 3 hr., the separatory funnel and connecting tubes are washed three times by pumping 20-ml. portions of warm distilled water through the assembly. Ashing is continued on the turntable for about 1 hr. and then the crucible is transferred to the muffle furnace for 12 to 24 hr., depending on the temperature required. To minimize losses of cesium, the temperature should not exceed 450° C. (8). If this element is of no interest, the temperature may be raised safely to 550–600° C.

RESULTS AND DISCUSSION

Types of crucibles for ashing milk. A suitable type of dish for the complete ashing operation had to be heat- and shock-resistant and one which would not interfere in the analysis of radionuclides. Iron, nickel, and "Vycor" silica crucibles were tried in the ashing process described. Iron vessels blistered, forming scale during the period in the muffle furnace, which contaminated the ash and interfered with the chemical separation of the radionuclides. The nickel crucibles fractured after one or two ashings, either during heating with the burner or in the muffle furnace, making quantitative collection of ash impossible. The "Vycor" silica dishes proved highly satisfactory, since they held up under the ashing operation and did not contribute materials to the sample which interfered with the subsequent analyses.

Comparison of the rapid with the conventional ashing method. To determine whether the rapid ashing method is satisfactory and comparable to the conventional methods, 1,000-ml. aliquots of several samples of fresh raw whole milk were ashed by the rapid procedure described. Fifty-milliliter aliquots of the same milk samples were also ashed in platinum crucibles, according to the method of Overman *et al.* (7). Calcium determinations on aliquots of the various ash solutions were made according to the procedure described by Overman *et al.* (7). The results are summarized in Table 1. Calcium content of milk was chosen as the basis for comparison, since it is a nonvolatile element and its concentration can be determined easily with a high degree of precision.

Statistical analyses of these data indicate that the calcium values obtained from ash by the rapid method are not significantly different from those obtained from ash prepared by the Overman (7) method.

Recovery of calcium added to milk by the rapid ashing method. To determine the accuracy of the rapid ashing method, a single sample of fresh raw whole milk was obtained and was divided into seven 1,000-ml. aliquots. Calcium in

TABLE 1
A comparison of the ashing methods for milk[a]

Rapid ashing method	Conventional ashing method
(calcium meq/l)	
56.06	56.77
52.30	52.32
56.66	56.45
55.28[b]	55.56
56.11	56.49
55.28	55.51

[a] Based on calcium content of milk.
[b] Average of five ashings.

Analysis of variance

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio	Significance
Method	1	0.139	0.139	2.25	N.S.
Samples	4	25.445	6.361	102.9	V.S.
Error	4	0.247	0.0618		
Total	9	25.831			

amounts of 4.81, 7.21, 9.62, 12.02, 14.42, and 19.23 meq. as solutions of calcium chloride were added to six of the aliquots, respectively, and were ashed in "Vycor" silica dishes as previously described. Calcium analyses were made on aliquots of the ash solutions and the results are presented in Table 2.

The data show that recovery of added calcium to milk is 100.17% ± 2.49% and is judged to be satisfactory for the purpose intended.

Application of the method to other dairy products. To determine the applicability of the rapid technique for ashing dairy products other than milk, tests were made on ice cream and coffee cream. About 1,000 g. of ice cream were

TABLE 2
Recovery of added calcium to milk by the rapid ashing method

Added	Found	Recovery
(calcium meq/l)		*(%)*
0.00	54.59	.....
4.81	59.45	101.04
7.21	61.84	100.56
9.62	64.62	104.26
12.02	66.26	97.09
14.42	68.75	98.20
19.23	73.81	99.90
		Av. 100.17 ± 2.49

TABLE 3
A comparison of ashing methods for dairy products[a]

Sample	Rapid ashing method	Conventional ashing method
	(calcium meq/1,000g.)	
Ice cream	79.07	79.22
Coffee cream	50.10	50.60

[a] Based on calcium content of milk.

weighed and melted, and quantitatively transferred to the separatory funnel. The sample was ashed as described before. Similarly, 1,000 g. of coffee cream were ashed. Calcium analyses on aliquots of the ash solution (Table 3) were compared with those obtained by the usual procedure and were found to be in good agreement.

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CHEMICAL STABILITY OF INDICATORS AND SUBSTRATE-INDICATORS USED FOR THE DETECTION OF PHOSPHATASE ACTIVITY

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SUMMARY

The reagents 2, 6-dibromo-N-chloro-*p*-quinoneimine (BQC), N, 2, 6-trichloro-*p*-quinoneimine (CQC), phenolphthalein phosphate, and *p*-nitrophenyl phosphate, in powder form and as solutions, for use as indicators or substrate-indicators in phosphatase testing, were stored under a variety of conditions, to observe rates of deterioration.

CQC reagent was remarkably superior to BQC in maintaining chemical stability over long periods of time. Storage conditions for maximum stability of the four reagents were determined. BQC and CQC powder may be stored at any temperature between −5 and 20° C. for as long as 1 yr. BQC solutions may be stored at −5° C. for not longer than 30 days. CQC solutions may be stored at 5° C. for as long as 258 days. Phenolphthalein phosphate powder may be stored at −5° C. as long as 1 yr., whereas solutions of this reagent may be stored at −5° C. as long as 258 days. Powdered *p*-nitrophenyl phosphate may be stored at −5° C. as long as 105 days, and solutions of this reagent may be stored at −5° C. as long as 81 days.

The indicators CQC and BQC, in solution, are best kept in dark brown, well-stoppered glass containers, while the two substrate-indicators are best protected in clear glass, well-stoppered containers.

CQC and BQC were compared relative to their solubilities and rate and intensity of color development in simple model systems and in actual milk phosphatase systems. In absolute methanol, absolute ethanol, and 95% ethanol, CQC was rapidly and completely soluble. BQC was readily soluble only in absolute methanol.

The rate of blue color development was influenced greatly by the chemical system in which the indicators reacted with available phenol. The rate was similar for both BQC and CQC in the zinc sulfate–borate color development system associated with the Sanders and Sager method (8). In the trichloracetic acid–carbonate buffer color development system of the Cornell phosphatase method, both indicators reacted more rapidly with the available phenol. Maximum color development for each indicator was attained four times faster at 37° C. and twice as fast at 25° C. in the trichloracetic acid–carbonate system. The data show that maximum color can be developed in the Cornell method in 15 min. at room temperature, rather than the earlier recommended 15 min. at 37° C.

In the trichloroacetic acid–carbonate system CQC developed slightly more blue color than BQC at high levels of free phenol, but at low levels no differences were observed.

The chemical stability, solubility, and rate and intensity of color development indicate that CQC is more suitable for use as an indicator for phosphatase testing than is BQC. It should receive preference in the future.

The phosphatase test developed by Kay and Graham (5) in 1935 for the detection of raw and underpasteurized milks utilized Folin's and Ciocalteau's reagent as an indicator of phenol. Later, because of the relative nonspecificity of this indicator for phenol, Scharer (11) substituted 2,6-dibromo-N-chloro-*p*-quinoneimine (BQC). The quinonechloroimines earlier had been shown by Gibbs (4) to combine with phenol to produce a deeply colored blue indophenol. Dihalogen-substituted quinonechloroimines, BQC and N, 2,6-trichloro-*p*-quinoneimine (CQC), were most sensitive and stable in their reactions with those phenols in which the position *para* to the hydroxyl group was unsubstituted (4). Before

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a method of testing for the efficiency of pasteurization was known, these indicators were found useful for detecting small amounts of phenol in water supplies.

In the two phosphatase methods most recently developed for all dairy products, the Sanders and Sager (9) and the Cornell (6) methods, the indicator BQC was designated. Both methods refer to the possible use of CQC. For example, Sanders and Sager favored CQC in their rapid field test for Cheddar cheese (8), but not in their laboratory method. It was pointed out that CQC appeared more stable and was less adversely affected by protein interference. Scharer (12) favored CQC because of better chemical stability. Despite this trend of thought, BQC is designated and used in most phosphatase methods and little emphasis has been placed on the use of CQC.

In certain modifications of the phosphatase test, substrate-indicators—forming characteristic colors in alkaline solution after hydrolysis by the phosphatase enzyme—may be used instead. Two examples of these substrate-indicators are phenophthalein phosphate and *p*-nitrophenyl phosphate, which form pink and yellow-green colors, respectively, in alkaline solution.

A matter of constant concern in phosphatase testing is the chemical stability of indicators or substrate-indicators. BQC powder, on standing, may change from the original lemon color to a brown. In this form, BQC loses the ability to form any, or at best maximum, blue color with free phenol (4). Little is known about the stability of CQC powder, but it appears to be more resistant to decomposition than BQC (8, 11). In solution, the chemical deterioration of the two indicators is conceivably accelerated. Instability of the substrate indicators presents a more acute problem. Here decomposition frees the indicator group within the molecule, spontaneously yielding the pink or yellow-green color, depending on which substrate-indicator is used. The decomposition of the substrate indicator makes the buffer-substrate as well as the indicator useless for the detection of the phosphatase enzyme.

Brief statements, in general terms, appear in the literature concerning the deterioration of these indicators (1, 2, 4, 8, 11, 12). A paucity of scientific data concerning the general merits of CQC and BQC, relative to time and conditions of decomposition and their behavior in the two phosphatase methods used for the testing of all dairy products, led to the present study.

METHODS

Powdered samples, from a single source, of the indicators CQC and BQC and of the substrate-indicators phenophthalein phosphate and *p*-nitrophenyl phosphate, were placed in storage at −5, 5, and 20° C. in both brown and clear glass containers, with and without desiccation. Observations on stability were made at regular intervals over a period of about 1 yr. Powdered BQC and CQC samples were periodically dissolved in absolute ethyl alcohol at a concentration of 0.017 g/3 ml. The prepared indicators were then tested in model systems consisting of 5 ml. of Cornell buffer (6) and 0.07 ml. of freshly prepared phenol solution (1 g/500 ml. water). Cornell buffer is prepared by dissolving 11.50 g. anhydrous Na_2CO_3 and 10.15 g. anyhydrous $NaHCO_3$ in distilled water and making up to a volume of one liter. Two drops of indicator, measured with a

dropper delivering 45 drops/ml, were added to the model system to produce the indophenol blue color. The substrate-indicator powders were removed from storage and dissolved in Cornell buffer at concentrations of 0.01 g. powder/15 ml. buffer.

Indicators and substrate-indicator solutions were stored at the same temperatures in brown and clear glass containers and examined for decomposition over a 9-mo. period in the same model systems. CQC and BQC were stored as alcoholic solutions at concentrations of 0.30 g/50 ml. of absolute ethyl alcohol. The substrate-indicators were stored in Cornell buffer at a concentration of 0.67 g/liter for phenolphthalein phosphate and 0.20 g/liter for *p*-nitrophenyl phosphate.

The light transmission of all solutions was measured in a Beckman Model DU quartz spectrophotometer, using Cornell buffer as 100% transmission. Measurements were made at the following wave lengths and slit widths: BQC and CQC, 590 mμ and 0.06 mm.; phenolphthalein phosphate, 555 mμ and 0.10 mm., and *p*-nitrophenyl phosphate, 390 mμ and 0.36 mm.

The sensitivity and behavior of CQC and BQC as indicators were observed in a variety of ways. Equimolar solutions of these indicators were prepared in absolute methanol, absolute ethanol, and 95% ethanol. For most applications, solutions with concentrations of 50 mg. BQC/10 ml. alcohol and 37 mg. of CQC/10 ml. of alcohol were studied. These solutions prior to use were kept at −5° C. and when needed for testing were dispensed with a dropper delivering 45 drops/ml. For comparison of rate and intensity of blue color produced in the presence of known concentration of phenol, two drops of indicator was added to 10 ml. of Cornell buffer containing graded amounts of phenol. Color was developed at 37° C. for 30 min.

To test indicator performance in milk containing active phosphatase, highly heated milks (180° F. for 5 min.) containing 0.0, 0.1, 0.2, 0.3, and 0.4% added raw milk were prepared. The rate and intensity of color developed, using BQC and CQC under conditions recommended for the Cornell method and the Sanders and Sager method (8), were followed with stop watch and spectrophotometer. Two drops of indicator were used in each method (excess). The transmission readings were made at 610 mμ and a slit width of 0.0575. These samples were transferred to cuvettes after the addition of the indicator at the proper temperatures and first readings were made at 5 min. Successive readings were made each minute until maximum color was developed. Maximum color was taken as the point at which successive readings indicated less than 0.5% change in transmission value.

Reagents were obtained from the designated source.[2] The indicators from several sources showed similar results, but the results presented are for the first source listed.

[2] CQC from Distillation Products Industries, Eastman Organic Chemicals Dept., Rochester 3, New York, and Applied Research Institute, 2 East 23rd St., New York 10, New York. BQC from same two sources. Phenolphthalein phosphate from Sigma Chemical Co., 4648 Easton Ave., St. Louis 13, Missouri. *p*-Nitrophenyl phosphate from Sigma Chemical Co.

RESULTS

Deterioration of indicators and substrate-indicators on storage. Decomposition of CQC and BQC is readily measured in model systems by a decrease in the indophenol blue color formed, resulting in an increase in light transmission values. Eventually, when decomposition is advanced, this blue color is completely dissipated and replaced by a brown color. Substrate-indicator decomposition was easily measured by the presence of pink or yellow-green color in the white powders or colorless solutions, resulting in a decrease in light transmission values.

Neither the BQC nor CQC powders displayed any signs of deterioration during the 1-yr. storage period under the conditions of storage employed (Table 1). The yellow color of both powders was maintained and the only evidence of

TABLE 1

Rate of chemical decomposition of powdered indicators, 2,6-dibromo-N-chloro-*p*-quinoneimine (BQC) and N, 2,6-trichloro-*p*-quinoneimine (CQC), used in phosphatase testing

Condition of storage	Time (*wk.*) 0	5	15	28	36	52
	Transmission values[a]					
(° C.)	(%)					
	2,6-Dibromo-N-chloro-*p*-quinoneimine (BQC)					
20 W	2.8	2.7	3.6	3.0	3.0	1.7
20 B	3.5	2.7	3.5	2.8	4.6	1.1
20 W–D	3.3	4.1	2.9	3.5	2.5	2.2
20 B–D	3.5	2.6	3.0	2.8	2.6	1.9
−5 W	2.7	3.0	2.4	2.7	3.3	2.1
5 W	3.7	3.1	2.9	2.5	2.3	1.6
5 B	2.7	3.5	3.1	2.3	2.1	1.9
5 W–D	3.1	2.2	3.3	3.5	2.0	2.1
5 B–D	2.9	2.5	2.9	3.0	2.8	2.5
	N,2,6-Trichloro-*p*-quinoneimine (CQC)					
20 W	1.8	2.3	2.3	1.1	2.2	1.6
20 B	1.8	1.8	2.6	1.3	2.0	1.3
20 W–D	2.3	2.7	2.3	1.7	1.9	1.0
20 B–D	1.8	2.2	2.2	1.1	1.8	1.4
−5 W	1.8	2.6	2.1	1.6	3.0	1.4
5 W	1.6	2.6	2.0	2.1	2.8	3.2
5 B	1.5	2.3	2.4	2.3	2.7	2.6
5 W–D	1.6	2.2	1.7	2.1	1.4	1.5
5 B–D	1.6	2.5	1.9	2.2	2.6	1.3

W—Colorless bottle, well-stoppered.
B—Brown bottle, well-stoppered.
D—Held in desiccator.
[a]—Decomposition increases with increase in per cent transmission.

change occurred in samples stored at −5° C. These samples became lumpy, due to moisture retention.

Storage of the alcoholic solutions of these same indicators produced marked changes, especially with BQC (Table 3). The zero-time measurement of freshly prepared BQC and CQC solutions gave light transmission readings of about 2%. At 20° C. BQC deterioration was rapid and in less than two days, in both types of glass containers, this indicator failed to develop any blue color in the model systems, whereas transmission values rose to 90%. Measurable deterioration

occurred in less than six days at 5° C. and in less than 53 days at −5° C., in both types of glass containers.

CQC solutions proved remarkably stable. In clear glass containers at 20° C., signs of deterioration were not evident until after 30 days; whereas, in brown glass containers CQC solutions were stable for 95 days. At 5 and −5° C. no deterioration was evident after 258 days, the length of time measurements were made.

TABLE 2

Rates of chemical decomposition of powdered substrate-indicators, phenolphthalein phosphate, and *p*-nitrophenyl phosphate, used in phosphatase testing

Condition of storage	Time *(wk.)* 0	5	11	15	20	24	28	36	52
	Transmission values*								
(°C)	*(%)*								
	Phenolphthalein phosphate (PPO_4)								
20 W	62.0	59.5	59.0	57.5	53.5	48.0	50.0	45.5	51.5
20 B	59.0	58.5	59.0	58.0	54.0	50.0	51.0	46.5	51.0
20 W–D	59.5	58.0	57.0	52.0	49.0	47.0	46.0	39.0	38.0
20 B–D	60.0	55.5	53.0	52.0	44.5	44.0	47.0	41.0	40.0
−5 W	60.5	61.0	61.0	61.5	56.5	59.5	60.0	60.5	61.0
5 W	60.0	61.5	64.5	.....	.....	.....	.....	.....	.....
5 B	59.5	62.5	62.0	63.5	57.5	64.0	63.5	.....	.....
5 W–D	61.0	60.5	60.0	62.0	57.5	59.0	58.0	57.0	59.0
5 B–D	60.0	59.5	57.0	57.0	52.5	57.5	55.0	55.0	55.5
	p-Nitrophenyl phosphate ($PNPPO_4$)								
20 W	75.0	72.0	67.0	62.5	57.0	42.0	61.0	47.0	60.5
20 B	77.0	73.5	67.0	61.0	51.0	40.5	61.0	57.5	63.0
20 W–D	75.5	63.0	38.5	26.0	26.0	5.3	18.5	32.0	18.0
20 B–D	76.0	64.0	44.0	23.0	24.0	20.0	20.0	31.0	18.0
−5 W	76.0	78.5	74.0	70.0	58.0	46.5	69.5	77.0	70.0
5 W	72.0	79.5	74.0	71.5	64.0	52.0	72.0	65.5	73.5
5 B	73.0	78.0	71.0	71.0	63.0	58.0	72.0	60.5	73.0
5 W–D	73.0	74.5	64.0	65.0	44.5	53.5	46.5	58.5	49.0
5 B–D	73.0	78.0	67.0	66.0	57.5	54.5	70.0	67.5	58.0

W—Colorless bottle, well-stoppered.
B—Brown bottle, well-stoppered.
D—Held in desiccator.
*—Decomposition increases with decrease in per cent transmission.

Phenolphthalein phosphate powder stored at 20° C. decomposed slowly over a period of weeks. Deterioration was hastened if it was stored in a desiccator, but light was without effect. At 5 and −5° C. no deterioration was evident after 1 yr. of storage. After 15 wk. of storage, the 5° C. sample became gummy and difficult to sample, due to moisture retention. Data are shown in Table 2.

Stored as a solution, phenolphthalein phosphate showed evidence of deterioration at 20° C. in less than 81 days in the clear glass containers and in similar containers covered with lead foil to exclude all light (Table 3). At 5° C. in clear glass containers decomposition was evident after 123 days and in lead foil–covered containers after 30 days. In the frozen state (−5° C.) phenolphthalein phosphate was stable after 258 days, the duration of the experiment.

The substrate-indicator, *p*-nitrophenyl phosphate, in powder form and in solution, was markedly less stable under all conditions of storage than phenolphthalein phosphate. Stored as a powder in a desiccator, it deteriorated rapidly,

TABLE 3

Rate of chemical deterioration of solutions of indicators and substrate-indicators used in phosphatase testing

Condition of storage		Time *(days)*														
		0	2	6	16	24	30	53	81	95	109	123	144	170	223	258
		Transmission values														
(°C.)		*(%)*														
		2,6-Dibromo-N-chloro-*p*-quinoneimine (BQC)*														
20	W	2.1	91.5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
20	B	1.9	91.5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	W	2.1	3.2	6.4	34.0	79.0	90.0	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	B	1.9	2.9	5.1	26.0	63.0	87.0	89.5	.....	.....	.....	.....	.....	.....	.....	.....
—5	W	1.9	2.2	2.5	2.9	3.4	3.5	5.0	6.8	8.1	12.0	13.0	15.5	24.0	47.0	68.0
		N,2,6-Trichloro-*p*-quinoneimine (CQC)*														
20	W	2.1	2.7	2.3	2.4	2.4	2.5	4.7	39.0	78.5	93.0	.....	.....	.....	.....	.....
20	B	2.4	2.2	2.4	2.1	2.1	1.5	1.0	1.7	2.6	7.3	28.0	83.5	90.0	.....	.....
5	W	2.3	2.0	2.2	2.3	.....	2.4	2.6	1.9	2.4	3.0	3.0	2.2	2.2	1.5	2.0
5	B	2.7	2.3	2.1	2.2	1.5	2.5	1.9	1.2	1.5	1.8	1.9	1.3	1.4	0.7	0.6
—5	W	2.1	1.7	1.9	2.5	2.1	1.9	1.9	2.9	2.2	3.0	2.2	2.0	2.8	2.1	2.3
		Phenolphthalein phosphate (PPO_4)**														
20	W	60.0	60.5	61.5	59.5	58.5	60.0	58.5	43.0	35.0	27.0	19.5	11.5	7.0	1.5	.....
20	B	60.0	60.0	61.5	58.5	56.5	57.0	55.5	53.0	52.0	51.0	49.5	49.0	46.5	44.0	45.0
5	W	60.0	60.0	60.0	60.0	59.0	60.5	60.0	59.0	59.0	58.5	58.0	55.5	38.0	21.0	15.0
5	B	60.0	60.0	61.0	58.5	56.0	57.0	53.0	49.0	47.0	45.5	43.0	41.0	37.5	24.5	11.0
—5	W	60.0	60.0	62.0	60.0	58.0	60.0	59.0	59.0	59.0	58.5	58.0	58.5	58.5	57.5	57.0
		p-Nitrophenyl phosphate ($PNPPO_4$)**														
20	W	71.5	71.5	74.0	70.0	65.0	63.0	51.5	33.0	28.0	25.0	18.0	8.7	3.0	.....	.....
20	B	71.5	69.0	67.0	55.0	47.5	44.5	23.0	4.2	1.6	.....	.....	.....	.....	.....	.....
5	W	71.5	72.0	75.0	71.0	69.0	71.0	67.5	62.0	60.0	60.0	55.0	47.0	23.5	1.0	.....
5	B	71.5	71.5	73.0	67.5	64.0	64.0	55.5	46.0	42.0	39.0	10.0	0.8	.....	.....	.....
—5	W	71.5	72.0	74.0	69.5	69.0	70.5	66.5	65.0	65.0	62.0	62.0	45.0	46.0	57.0	54.0

W—Colorless bottle, well-stoppered.
B—Brown bottle, well-stoppered.
D—Held in desiccator.
..... —Samples completely decomposed.
*—Decomposition increases with increase in per cent transmission.
**—Decomposition increases with decrease in per cent transmission.

probably due to loss of water of hydration. This reaction may account for the erratic transmission values for this substrate-indicator after 24 wk. of storage as a powder, as shown in Table 2. The stability at 5° C. of the powder confirms the observations of Aschaffenburg (1).

At 20° C. the *p*-nitrophenyl phosphate solutions deteriorated in less than 24 days in clear glass containers and in less than 16 days in foil-covered containers (Table 3). At 5° C. deterioration occurred in less than 81 days in clear glass containers and in less than 24 days in foil-covered containers. Freezing protected the substrate-indicator for about 53 days.

Solubility of indicators in alcohols. The indicator, CQC, in concentrations of 250 mg. solute per 50 ml. of solvent at 23° C. was completely and readily soluble in absolute methanol, absolute ethanol, and 95% ethanol, requiring only from 2 to 6 min. to attain this state. BQC, on the other hand, was readily soluble only in absolute methanol. Despite the aid of mechanical grinding, BQC dissolved in absolute ethanol only after 60 min.; whereas, in 95% ethanol it was not dissolved completely after 24 hr.

Blue color development in model buffer system containing added phenol. The ability of CQC and BQC to produce color in 30 min. at 37° C. was tested in prepared buffer systems (pH 9.7) containing 1, 2, 4, and 8 γ phenol. Color maximums for each indicator, at each phenol level and in three alcoholic solvents were similar, indicating that in a simple model system devoid of protein or protein products, CQC and BQC, regardless of solvent, will react with known amounts of phenol to give the same intensity of color.

Color produced by CQC and BQC with different levels of raw milk contamination in pasteurized milks. A highly heated negative control milk was contaminated with levels of raw milk ranging from 0.1 to 0.4%. Split samples of milk were tested with the 18-hr. Cornell method, using two drops of either BQC or CQC as indicator. Color was developed at 37° C. for 15 min.

At the 0.1% level of raw milk contamination the same intensity of color was produced by both indicators. With milks of greater raw contamination (0.2 to 0.4%), CQC produced a greater intensity of blue color than did BQC (Table 4). This increase, though not marked, seemed significant. As model systems showed no differences between the intensity of color produced by the two indicators, it is assumed that protein components from the milk influenced the effectiveness of CQC in the production of greater intensities of color.

Rate of color development as influenced by temperature and the type of phosphatase test. The times required for maximum color development at 25 and 37° C., using CQC and BQC, are presented in Table 5. Since the Sanders and Sager test (8) employs widely different chemicals than the Cornell method, for precipitation and for color development, these two different systems may influence the rate of color development. Temperature of color development had relatively little effect on color development with the Sanders and Sager system. Maximum color had developed in 30 min. at 25° C. and in 25 min. at 37° C. with BQC. When CQC was used under similar conditions, a slightly greater time for color development was required, 40 min. at 25° C. and 30 min. at 37° C.

TABLE 4

Transmission values for milks containing 0.1–0.4% raw and incubated at 37° C. using BQC and CQC as indicator[a]

(Average 12 trials)

Sample	BQC	CQC
Highly heated blank	75.0	75.0
0.1% Raw	62.0	62.0
0.2% Raw	54.0	52.0
0.3% Raw	49.0	45.0
0.4% Raw	44.0	38.0

[a] In trichloracetic acid–carbonate buffer color development system.

TABLE 5

Time and intensity of maximum color development using BQC and CQC as indicator in two buffer color development systems at two temperatures

(Two drops each indicator)

(Average two determinations)

Per cent raw added	Time *(min.)*	Per cent T	Phenol γ	Time *(min.)*	Per cent T	Phenol γ
			Zinc sulfate–borate system[a]			
			25° C.			
		BQC			CQC	
0.1	30	69.5	6	40	75.0	5
0.3	25	58.5	8	35	62.5	8
0.5	25	50.0	13	25	52.0	13
			37° C.			
0.1	20	69.0	5	30	74.5	4
0.3	25	58.0	10	30	61.5	10
0.5	25	50.0	14	25	51.0	14
			Trichloracetic acid–carbonate system[b]			
			25° C.			
		BQC			CQC	
0.1	15	55.5	12	15	52.0	13
0.3	15	34.0	24	10	19.0	40
0.5	10	29.0	32	10	11.5	>40
			37° C.			
0.1	5	58.5	10	15	52.5	13
0.3	6	39.5	19	7	21.0	36
0.5	5	32.5	28	4	14.0	>40

γ From visual color standards. All 37° C. tests dropped to 34–35° C. in 30 min.

[a] Sanders-Sager Method, pH 9.4.

[b] Cornell Method, pH 9.7.

With the Cornell phosphatase method color development system, the blue color development was significantly faster at either of the two temperatures (Table 5). For example, maximum color development using BQC was attained in 15 min. at 25° C. and in 6 min. at 37° C. Using CQC, a similar fast rate was recorded with the Cornell method. In addition, CQC gave a deeper blue color with the same milks than did BQC, but this phenomenon was restricted to the Cornell method, a confirmation of earlier observations made with model systems. Another interesting observation is that maximum color development is achieved faster with larger amounts of free phenol present. These data indicate clearly that the rate of color development with BQC and CQC is not only a function of

temperature but also of the nature of the color development system and perhaps of other factors.

DISCUSSION

Though Gibbs (4) found that BQC crystals undergo slight decomposition on standing, resulting in brown colors, none of the BQC or CQC powders used in this work turned brown. Perhaps if a storage period of greater than 1 yr. had been used, a change in color might have occurred, especially if moisture had been present. One sample of CQC powder (18 were stored) turned black during storage and when dissolved in absolute ethanol yielded a brown solution. Its ability to form blue indophenols was unimpaired. This color change in the powdered form was probably due to an impurity rather than a change in the indicator

Other investigators (2, 3, 8, 10, 11, 12) have been aware of the instability of BQC solutions and these views were confirmed in this work. The remarkable stability of CQC solutions under storage conditions adverse for BQC has escaped general notice.

Little information is available concerning factors affecting the stability of phenolphthalein phosphate, but Aschaffenburg (1) had observed that storage exposed to light in a dry state adversely affected *p*-nitrophenyl phosphate powders. His well-stoppered, air-dried preparations were maintained for years without forming yellow colors, in both cold rooms and at room temperature, without evidence of deterioration, if protected from strong light. In the present work, storage of powders in a desiccated state led to more rapid deterioration of the two substrate-indicators, but the solutions of these chemicals were provided better protection when stored under natural light than when stored in foil-covered containers which excluded all light.

Phenolphthalein phosphate, BQC, and CQC powders showed hygroscopic properties when held at 5° C., which resulted in gumminess, making it difficult to sample these preparations. Presumably, moisture from warm air entered the tightly stoppered containers when they were removed from storage for sampling. Solutions of *p*-nitrophenyl phosphate began to show an erratic transmission pattern after 144 days of storage at −5° C. (Table 3). This may have been due to the slight solubility of the free indicator at room temperature, which removes yellow-green color from the solutions when the indicator exceeds its solubility. The erratic transmission patterns of the powders late in the storage period, presumably due to loss of water of hydration, have been discussed under Results.

The choice of an indicator for phosphatase testing may have an important bearing on the success of the finished test. Earlier literature indicates that CQC is preferable to BQC, despite the fact that BQC is most often recommended. Other attributes of these indicators have never been investigated. For example, a cursory observation commonly noted is that BQC, when added in amounts of from two to six drops to phosphatase filtrates containing very small amounts of free phenol, often turns reddish-brown. CQC, on the other hand, under similar circumstances creates little or no problem in this regard. Apparently, the differ-

ence in behavior is related to indicator stability in aqueous solutions and, perhaps, to the presence of soluble protein components in the filtrate.

Observations of Sanders and Sager (9), that maximum color development in their method when phenol and BQC react requires 30 min. at room temperature, were confirmed. It was observed that maximum color development was achieved significantly sooner in the chemical system of the Cornell method. At 37° C., maximum color was produced four times faster and at 25° C. twice as fast. This does not affect the sensitivity of either method, but the information permits color development in the Cornell method to proceed at room temperature for only 15 min., a simpler arrangement for the test.

The reasons why the rate of color development is speeded so dramatically in the Cornell method is not fully understood, but may be a result of the greater efficiency of protein precipitation or the result of a catalytic effect of trichloroacetic acid and/or carbonates used in this method.

Attention should be directed to an observation made in the course of the present study which explains why color does not fully develop in some phosphatase tests. A similar observation in the phosphatase testing of cheese has been recorded by Mahon *et al.* (7). When either indicator is added dropwise directly to the filtrate a brown discoloration appears at the surface. Quantitative measurements indicate that coincidently less blue color is realized per unit of phenol, particularly when BQC is used, and suggests some decomposition of the indicator in the aqueous solution before reaction with phenol. Complete color development and production is obtained when the indicator is added while the filtrate is being agitated. A simpler procedure, which accomplishes the desired result, is the pouring of the filtrate into a dry test tube containing the required number of drops of indicator.

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FACTORS INFLUENCING METABOLIC ACTIVITY OF BULL SPERMATOZOA. IV. pH, OSMOTIC PRESSURE, AND THE CATIONS, SODIUM, POTASSIUM, AND CALCIUM

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SUMMARY

The combined effects of increasing pH from 4 to 8, of increasing potassium concentration, at the expense of sodium, and of varying osmotic pressure on the aerobic metabolic responses of washed bovine spermatozoa provided with fructose have been determined in one experiment. Low pH and high potassium levels were found to be inhibitory of all measures of metabolism (oxygen uptake, fructose utilization, and lactic acid accumulation). Osmotic pressure had no significant effect. In a second experiment, variations in the calcium level of the diluent replaced the variations in the osmotic pressure. The inhibitory effect of potassium was eliminated by the presence of calcium. Consequently, pH was the only variable affecting metabolic response. The uptake of fructose in the first few minutes after resuspension of the washed cells in a fructose-containing diluent is more rapid if the diluent has a pH of 5 than if it has a pH of 7.5, though on continued exposure the utilization ceases at the low pH but continues unabated at the higher one.

Optimum preservation of the fertility function of spermatozoa after ejaculation is dependent upon reduction of their metabolic activity to minimum levels during storage and complete reversal of the inhibitory processes at insemination. Temperature control has been the inhibitory method of choice, with the diluent designed to provide protection against cold, and at body temperature to provide optimum conditions for motility and metabolic activity (4). These techniques have provided the basic advances responsible for the development of commercial artificial insemination of cattle since the discovery by Phillips (12) of the value of hens'-egg yolk for these purposes in 1939.

However, observations made in a number of laboratories in the course of studies on the epididymis and in measurements of metabolic activity of spermatozoa have indicated that other means of metabolic control were possible. These include the finding of a metabolic regulator in epididymal spermatozoa (9), of a metabolic inhibitor in ejaculated semen closely associated with the spermatozoa (2), and of the effects on metabolism of pH (10, 16), osmotic pressure (17), of the concentration of the cations, calcium and potassium (10, 22), the concentration of anions (3, 17), and of the partial pressure of carbon dioxide (18, 19). In addition, new evidence on the environment afforded spermatozoa in the epididymis, where they are normally quiescent, shows that the fluids are higher in potassium, lower in sodium and in calcium concentration than seminal plasma (7, 15).

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The independent effects of some of these factors on aerobic metabolism of spermatozoa have been reported. However, no data are available on the effect of a combination of some of them on the several measures of metabolic activity. The experiments reported here were conducted to determine the interacting effects, if they existed, of pH, osmotic pressure, calcium concentration, and potassium concentration on the aerobic metabolism of washed bovine sperm cells. A preliminary report of these experiments appeared in 1957 (6).

METHODS AND MATERIALS

Semen used in these experiments was collected from mature dairy bulls by an artificial vagina. The spermatozoa were twice washed with 0.154 *M* NaCl (saline), using the following procedure: The raw semen was diluted to approximately twice the original ejaculate volume with saline. The spermatozoa were then centrifuged out of suspension and the supernatant removed. The cells were suspended again in saline to a volume equal to the original ejaculate, recentrifuged, the supernatant removed, and the cells resuspended in saline solution to the desired concentration of cells. Two-tenths milliliter of this cell suspension was added to 0.8 ml. of test solution containing 200 mg/100 ml. of fructose as substrate in the Warburg reaction flasks. Oxygen uptake, fructose utilization, and lactic acid accumulation were measured during a 2-hr. incubation period at 37° C. Initial and final pH and motility of the suspended cells were recorded.

Fructose was determined by the method of Roe (14), as modified by Mann (11). Lactic acid was determined by the method of Barker and Summerson (1).

Three experiments were conducted in this study. The first two were three-factor composite type experiments (5) involving eight replications each. In Experiment One, the variables of pH, osmotic pressure, and potassium concentration were studied, whereas in the second experiment, the effects of pH, calcium concentration, and potassium concentration were studied. In these composite experiments, initial pH values of 4, 5, 6, 7, and 8 were obtained by combinations of citric acid and sodium bicarbonate. Molarities of the final diluents (before adding spermatozoa) expressed as NaCl were 0.084, 0.119, 0.154, 0.189, and 0.224 *M*. The theoretical osmotic pressure equivalents are approximately 195 to 417 milliosmols, assuming complete dissociation of ions. (A solution which has an osmotic pressure of 290 milliosmols or a 0.154 *M* NaCl solution is isotonic and has approximately the same freezing point depression as seminal plasma.) The actual osmotic pressures were 150 to 392 milliosmols. Potassium was included in the experiments in levels of 40, 120, 200, 280, and 360 mg. % final dilution. Calcium was included in the second experiment at levels of 2, 12, 22, 32, and 42 mg. % final dilution. Potassium or calcium additions were made at the expense of sodium. $MgCl_2 \cdot 6\,H_2O$ was included in all of the diluents at the rate of 0.5 ml. of a 1.54 *M* solution in 50 ml. of diluent.

The third aerobic experiment was a 3^2 factorial involving five replications, with the objective of determining the relationship of pH to metabolic activity at various intervals. The pH levels studied were 5, 6.25, and 7.5. The intervals at which samples were taken for fructose and lactic acid measurements were

0, 1, and 2 hr. A 10-min. period for equilibration of the spermatozoa and diluent preceded 0 hr. In this experiment, therefore, fructose utilization and lactic acid accumulation were measured to include the 10-min. equilibration period, whereas O_2 uptake measurements began at 0 hr. Fifty per cent of the diluent used in this experiment was made up with a combination of $Na_2HPO_4 \cdot 12\ H_2O$ and $NaH_2PO_4 \cdot H_2O$, depending on the pH desired; whereas, the other half contained 0.154 *M* NaCl with 0.5 ml. of 0.11 *M* $MgCl_2 \cdot 6\ H_2O$ per 50 ml. and fructose solutions (200 mg. % final dilution).

In the composite experiments, the metabolic responses are expressed as the conventional *Z* values (13) whereas, in Experiment 3, they are expressed as total microliters or micrograms/10^8 spermatozoa.

RESULTS

The results of the first experiment involving five pH levels, five levels of osmotic pressure, and five potassium concentrations are given in Figure 1. By

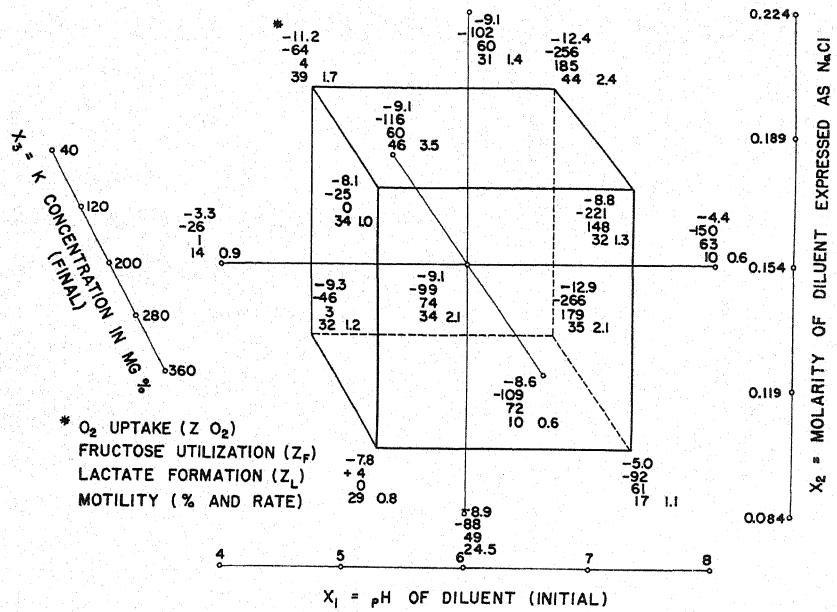


FIG. 1. Effects of pH, molarity of diluent, and potassium concentration on spermatozoan activity (average of eight responses).

study of this figure, one notes that the lower values of fructose utilization and of lactic acid production tend to be on the lower left side of the figure, at a low pH, a low osmotic pressure, and a high concentration of potassium. Statistical analysis of the combined data show that not all of the differences shown in the figure are significant ones. For example, considering the oxygen uptake only, the effect of pH was pronounced. The $Z\text{-}o_2$ was small at pH 4, increasing to a maximum in the center of the figure and decreasing again at the highest pH. This trend is

best fitted by a quadratic expression. The potassium level also influenced the oxygen uptake. The effect was least at low concentrations of potassium and greatest with the higher concentrations, but varying in degree with the pH level. On the other hand, the molarity of the diluent did not exhibit a significant effect on oxygen uptake.

The metabolic behavior of the washed spermatozoa as influenced by the three controlled variables can be expressed by regression equations, where X_1 is the effect of pH; X_2, that of varying molarity of the solutions; and X_3, that of potassium concentration. Only those regression coefficients (in either Experiment 1 or 2) to which a significant sum of squares could be attributed are given in the following equations:

$$Z_{O_2} = -10.6 + 1.1\,X_3 + 1.5\,X_1^2$$
$$Z_F = -119.8 - 60.2\,X_1 + 19.9\,X_3$$
$$Z_L = 88.5 + 43.1\,X_1 - 18.4\,X_1X_3$$

The five levels for pH (X_1) and the five levels for potassium (X_3) have been coded as −2, −1, 0, 1, and 2, respectively. For example, at a potassium content of 200 mg. % ($X_3 = 0$) and a pH of 5 ($X_1 = -1$), one would expect a Z_{O_2} of −9.1. (Instead of the usual plus values of Z_{O_2}, minus values are used here to indicate a utilization of O_2.) By use of these equations, estimates of response can be made for any set of conditions of the variables and ranges studied. The equations indicate that for fructose utilization, the effects of pH and of potassium concentration are linear and independent. However, for lactic acid accumulation, in addition to a pH effect, a significant interaction between pH and potassium concentration was found.

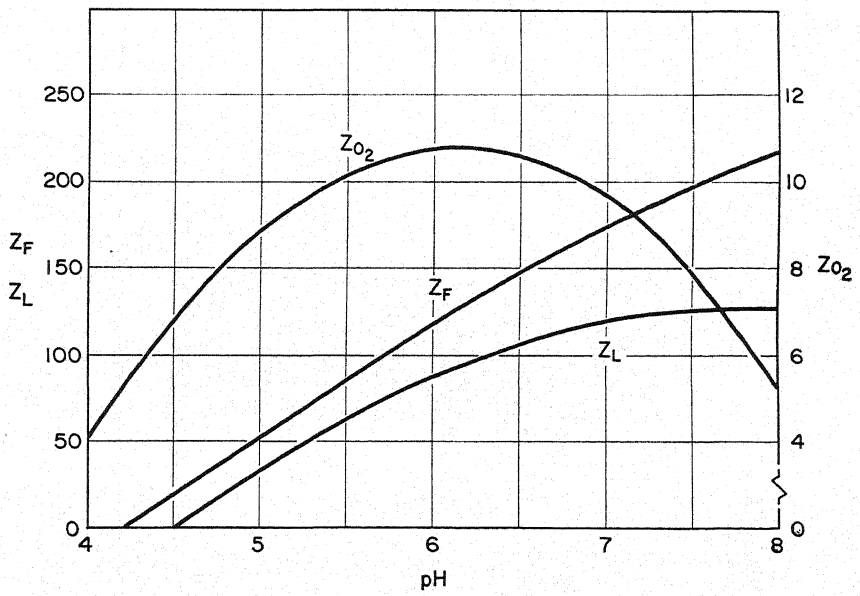


FIG. 2. The effect of pH on metabolic activity.

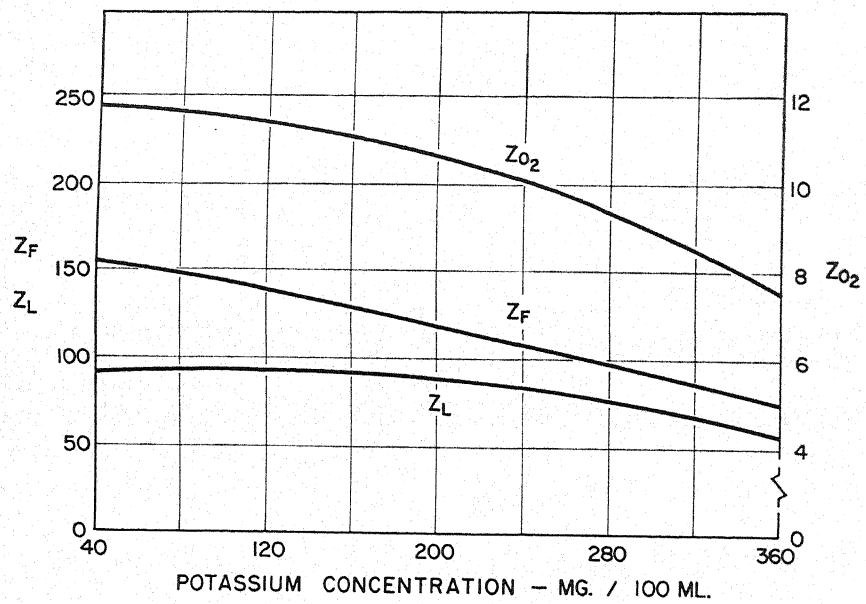


FIG. 3. The effect of potassium concentration on metabolic activity.

The primary effects of pH, of osmotic pressure, and of potassium concentration on the three metabolic responses in this experiment are illustrated in Figures 2, 3, and 4.

The combined results of the second experiment involving pH (X_1), calcium concentration (X_2), and potassium concentration (X_3) are given in Figure 5.

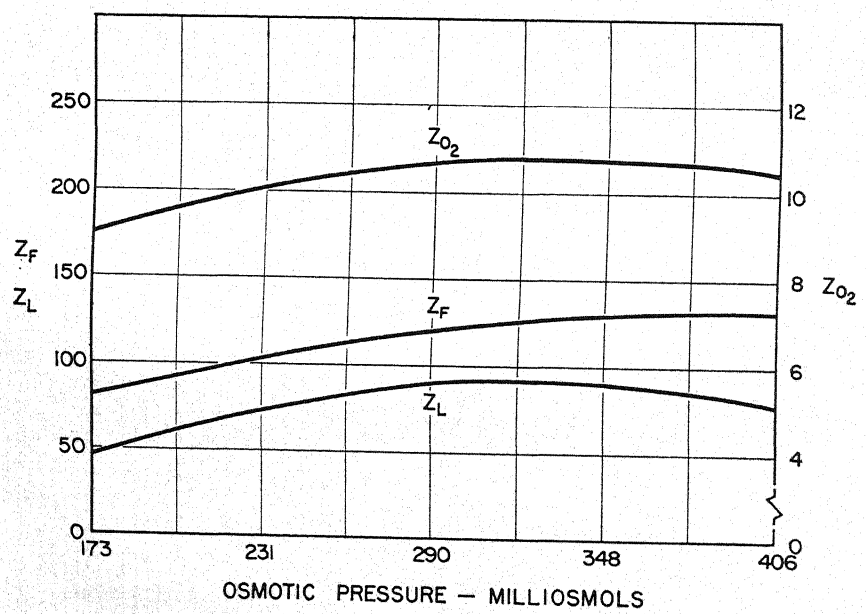


FIG. 4. The effect of osmotic pressure on metabolic activity.

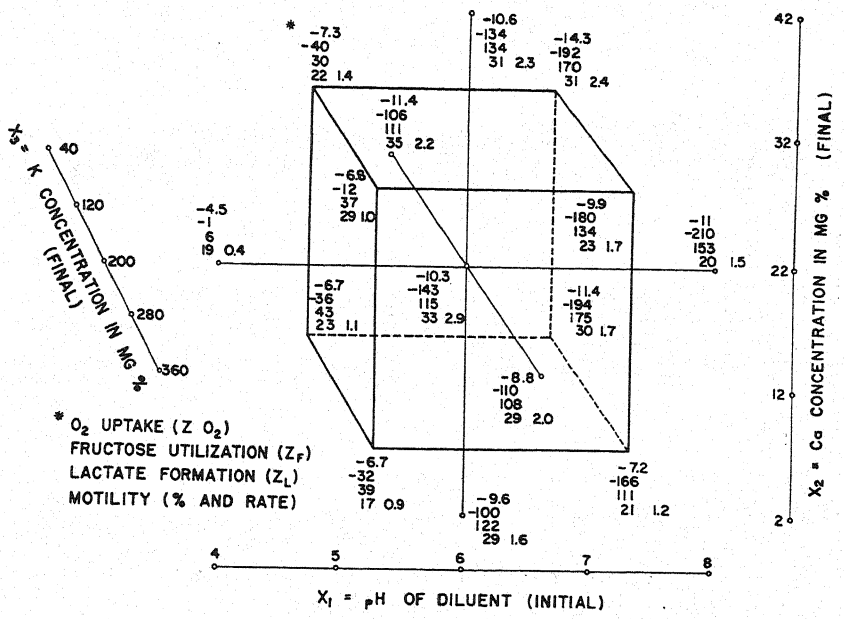


FIG. 5. Effects of pH, calcium concentration, and potassium concentration on spermatozoan activity (average of eight responses).

In this experiment, an interesting fact appears. Calcium not only has no marked effect on the metabolic response of the cells, but the previously observed marked inhibitory effects of increasing the concentration of potassium are virtually eliminated by its presence. Thus, in the regression equations derived from the second experiment, only the effects of pH (X_1) are included, for no other effect contributed a significant portion of the sum of squares.

$$Z_{O_2} = -9.6 - 1.8\,X_1$$
$$Z_F = -132.7 - 64.4\,X_1$$
$$Z_L = 101.4 + 46.0\,X_1$$

The effect of calcium concentration as found in this experiment is illustrated in Figure 6. The curves are drawn from the equations calculated for linear and quadratic effects, none of which was significant, so that the effects could equally as well be represented by straight lines across the chart representing the means for each metabolic measure.

The third experiment was designed to cast some further light on the rate of substrate uptake, as influenced by pH during the period of time in which temperature and gas pressure equilibration in the manometers and flask contents took place. The results are given in Table 1. The F values and the significant levels of the responses are given in Table 2.

The highly significant interactions of the linear effects of time and the linear effects of pH for both fructose utilization and lactic acid accumulation are the result of the fact that spermatozoa at pH 5 in a phosphate buffer take up a sizable

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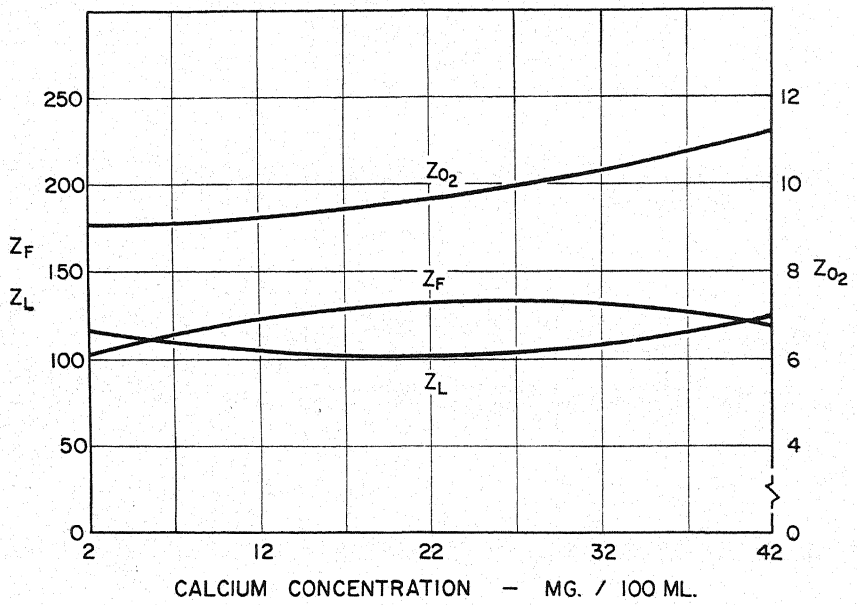


FIG. 6. The effects of calcium concentration on metabolic activity.

TABLE 1

Total oxygen uptake, fructose utilization, and lactic acid accumulation per 10^8 spermatozoa as influenced by time and pH in phosphate buffer (Experiment 3, mean of five replications)

Time	pH	O_2	Fructose	Lactate
		(μl.)	*μg.*	*μg.*
During equilibration period of 10 min.	5.00	.....	77	2
	6.25	.....	47	5
	7.50	.....	53	6
End of 1 hr. after equilibration	5.00	8.6	71	3
	6.25	9.4	67	27
	7.50	10.3	92	24
End of 2 hr. after equilibration	5.00	13.4	69	10
	6.25	12.7	79	34
	7.50	17.1	163	40

TABLE 2

Analysis of variance of data from Experiment 3 (F values)

	O_2 uptake	Fructose utilization	Lactic Acid accumulation
Time l[a]	179.5**	8.86**	59.38**
Time q[b]	5.66*	NS	NS
pH l	NS	NS	35.81**
pH q	NS	NS	8.03**
Time l × pH l	NS	10.28**	11.20**

* $P < .05$.
** $P < .01$.
[a] l = linear.
[b] q = quadratic.

quantity of fructose in the initial 10 min. (Time 0) of exposure to substrate. Thereafter, the substrate is metabolized at a slow rate, whereas at a higher pH, they exhibited much higher metabolic responses after a slower initial uptake of substrate.

These results show clearly that spermatozoa removal from the seminal plasma soon after collection, and which have undergone a period of substrate deprivation during washing and the preparatory stages of the experiment, take up hexose substrate at an initial rate substantially greater than the utilization rate. These results also show that at the lowest pH, after the initial uptake, which occurred within 10 min. and possibly before the intracellular pH was reduced below that of normal semen, no uptake occurs. These facts have great significance in the interpretation of other experiments and in the design of future ones.

DISCUSSION

A number of difficulties face investigators in this area of research. An important one deals with the relation of metabolism to visual estimates of spermatozoan motility. Our interpretation has been that if spermatozoan metabolic activity was inhibited and final motility was inhibited to a similar degree, no logical conclusion could be reached. Either inhibition and/or death of the cells may have taken place. Only in cases where final motility is the same as the control or only slightly depressed, and the metabolic measures are substantially decreased, can we conclude that metabolic inhibition has actually taken place.

A second, equally fundamental difficulty has been the large coefficients of variation in substrate utilization associated with experiments of this nature preventing precise measurement of treatment effects. In the first and second of these experiments, the coefficients of variation for fructose utilization were 40% or higher before ejaculate effects were removed. From the third experiment, it would appear that the preconditioning of spermatozoa as affected by the time from ejaculation to removal of the cells from the seminal plasma, the initial pH of the semen and, possibly, many more such factors, are concerned in such variability.

A third is a question primarily of semantics. In the first two experiments, the predominating cations in the diluents were sodium and potassium. When potassium was increased the sodium was decreased, and vice versa. This leaves the question of which ion should be considered as having the primary effect and the naming of that effect. We have chosen to consider the primary effect as due to potassium, because of its higher concentration in the cells (7) and the fact that the response of the cells to extracellular potassium was the one sought, namely, reversible inhibition of metabolic activity. The reversal of motility occurred upon removal of the cells from the Warburg flasks, dilution with 0.9% NaCl, and placing them under a cover slip on a microscope slide.

Low concentrations of potassium have been shown to be necessary for optimum metabolism of spermatozoa (22); whereas, high concentrations have been reported as detrimental (23). It now appears that in the absence of calcium (Experiment 1), reversible inhibition of metabolic activity at 37° C. takes place

at potassium concentrations of from 200 to 280 mg. %. The inhibition is particularly evident at pH 5, but the *K*-level × pH interaction was statistically significant only for the lactic acid accumulation. These potassium levels are higher than are reported for seminal plasma (a mean of 161 mg. %) (8), but not so high as are the amounts found in the epididymis, where the cells are normally quiescent (15). However, in the presence of calcium, the high potassium levels do not cause any measurable effect on sperm cell metabolism (Experiment 2). It does have a deleterious effect on motility. The interference of calcium with the inhibitory effects of potassium may have its counterpart in nature. The epididymal secretions are high in potassium and quite low in calcium; whereas, on ejaculaton, the high potassium is diluted with sodium and the calcium level is increased by addition of other products of the accessory glands, thus paving the way for the burst of activity associated with ejaculated semen.

The inhibition of metabolic activity by the potassium ion strongly suggests that this is at least one of the agents, if not the only one, responsible for depressing the oxygen uptake in whole semen reported by Bishop and Salisbury (2). These workers presented evidence that the inhibitor was associated with the spermatozoa. Subsequent work has shown that potassium is more concentrated within the spermatozoa than it is in the seminal plasma (8).

Finally, the sodium/potassium ratio deserves some consideration in view of the emphasis placed on it by other workers with bull spermatozoa (15, 21) and in the inhibition of trout sperm (20). It appears from the results reported here that the sodium/potassium ratio is a useful expression only under specified conditions of pH, low levels of calcium, and where sodium and potassium make up nearly all of the cations present.

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USE OF FLUORESCENT DYES FOR OBSERVING BOVINE SPERMATOZOA IN OPAQUE MEDIA

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SUMMARY

A method for staining sperm with fluorescent dyes and examination with ultraviolet light is presented. By using this method with semen diluted with opaque media, the spermatozoa were made clearly visible with no interference from fat globules or other extraneous material, and evaluation of sperm motility was readily made. These dyes in concentrations of 1:10,000 to 1:40,000 were nontoxic to the spermatozoa during the 15-min. incubation at 37° C. and caused only a slight reduction in motility after storage at 5° C. for 24 hr. The staining of sperm so that they fluoresce in ultraviolet light was found to make the sperm clearly visible for evaluation of motility in whole milk, yolk-phosphate, or other diluents in which white light illumination does not allow good discrimination between sperm and media. Other possible uses of this procedure also are discussed.

The observation and evaluation of bovine spermatozoa in diluents such as whole milk and yolk-phosphate have always presented a problem, because of the opacity of the media due to the presence of large numbers of interfering fat globules (4, 7). In an attempt to make the sperm more visible in such media, a study was conducted on the practicality of the routine staining of sperm with dyes which would cause the spermatozoa to fluoresce in ultraviolet light.

Fluorescent dyes for staining of spermatozoa have been utilized by a number of authors in the study of sperm morphology (1, 2, 3, 5). Bishop and Austin (2) reported that a spectacular polychromatic fluorescence, which clearly defined the acrosome and nucleus of the sperm, was obtained by staining fresh, living rodent spermatozoa with acridine orange, but less successful definition of sperm structures was obtained with rabbit and bull spermatozoa. Bishop (1) was able to differentiate between live and dead spermatozoa by fluorescence microscopy after staining with primulin and rhodamin G. Stockinger (5) obtained good fluorescence of human sperm after staining with acridine orange, but stated that this method could not be used for practical examination of sperm livability. On the other hand, Strugger and Rosenberger (6) claimed that the motility of goat sperm was undisturbed by staining with acridine orange. The latter also report that 21 of 27 goats artificially inseminated with spermatozoa stained with fluorochromes became pregnant and gave birth to normal young. More recently, workers in this laboratory have used the technique of staining with fluorescent dyes to make spermatozoa more visible when using visual methods (9), and to eliminate counting of fat particles and other extraneous material in milk diluents when using electronic methods of evaluating sperm motility (8).

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EXPERIMENTAL PROCEDURE

Fourteen dyes known for their ability to stain biological materials and fluoresce under ultraviolet light were studied for their applicability to the staining of living bovine spermatozoa. These dyes were arbitrarily rated from 1 to 6, depending on the sharpness of the fluorescence of the sperm cells and the absence of background illumination. These dyes are listed in Table 1, in the order of their decreasing acceptability for use in three different diluents at a final dilution of 1:20,000. Coriphosphin gave the best all-around results and is the dye of preference in this laboratory, although euchrysin 2, acridine orange, and akridingelb also rated high and gave satisfactory results in most cases.

A dye solution was prepared by dissolving one part of the dye in 1,000 or 2,000 parts warm 0.9% saline. This solution was filtered to remove any large undissolved particles of the dye and the pH was adjusted to 6.8 with 0.1 *N* NaOH.

This solution was then added to the diluted semen, both being at the same temperature, to give a final concentration of dye to diluent of between 1:10,000 and 1:40,000, and thoroughly mixed by inversion. A concentration of 1:20,000—25,000 gave maximum fluorescence of spermatozoa and minimum background illumination. The sperm took up the dye very readily, so that motility evaluation of the fluorescing sperm could be made immediately. However, best staining results were obtained if the samples were incubated in a water bath at 37° C. for 15 min. after addition of the dye solution.

A drop of the stained semen was then placed on a glass slide, covered with a cover slip, and examined under the microscope with ultraviolet light illumination in a darkened room.

Figure 1 illustrates diagrammatically the equipment needed for observation of the fluorescent sperm and the path of the light rays through the microscope.

TABLE 1

Average rating of fluorescence of sperm after staining with fluorochromes[b] diluted 1: 20,000 in three media

(Mean of ten or more samples)

Dye[b]	Diluent		
	Skimmilk	EYC[a]	Whole milk
Coriphosphin H K	5.0	4.0	6.0
Euchrysin 2 GNX	5.0	2.0	6.0
Acridine Orange	4.0	3.0	3.0
Akridingelb Extra	4.0	4.0	
Akriflavin HCl NF	3.1	3.0	2.0
Aurophosphin	3.5	0	
Auramin	1.7	1.0	
Thioflavin S	1.5	0	
Primulin O	1.0	1.0	
Berberin Sulfate Neutral	0.6	1.0	
Morin	0.3	0	
Berberin	0.1	0	
Brillantdianilgrun	0.1	0	
Fuchsin	0	0	

[a] One part egg yolk and one part 2.9% sodium citrate dihydrate.

[b] Manufactured by G. Grubler and Co., Berlin; George T. Gurr, Ltd., London; National Aniline Division, New York; New York Quinine and Chemical Works, Inc., New York, and generously supplied by Allen B. Dumont Laboratories, Clifton, New Jersey.

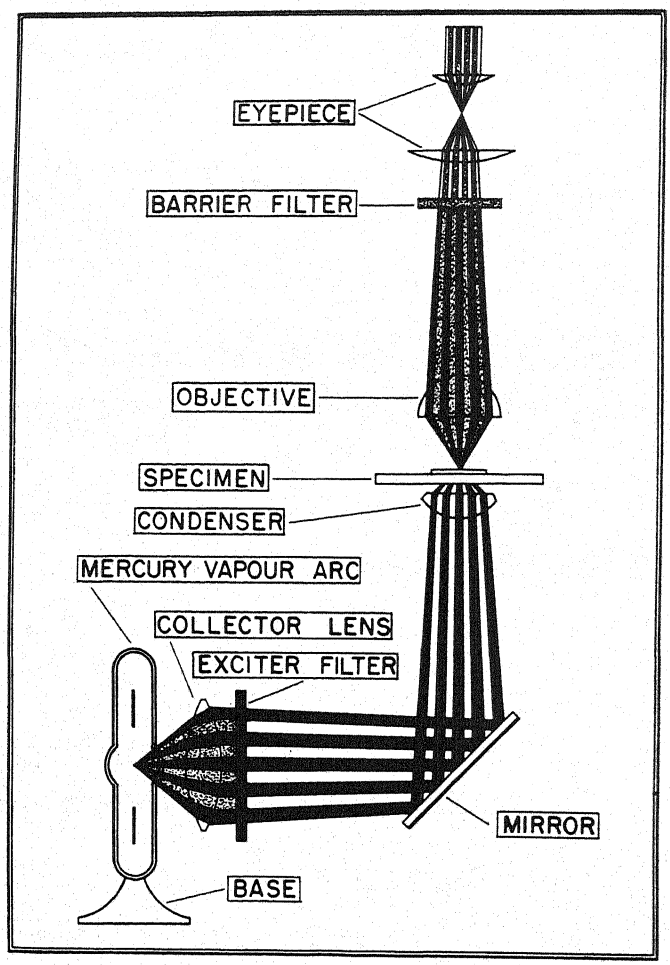


FIG. 1. A schematic diagram of equipment needed for observing sperm stained with fluorochromes. The solid lines represent ultraviolet light rays and the stippled lines represent visible light rays.

The ultraviolet light was provided by a mercury vapor lamp (G.E. AH-4) in a microscope spot lamp fitted with an exciter filter, Corning No. 5113, which transmits in the ultraviolet range. A barrier filter (Corning No. 3484 or American Optical No. 363-602) was placed in the eye-piece to absorb the ultraviolet rays while allowing passage of the visible light emitted by the fluorescing specimen. The percentage of motile sperm was determined and the motility was rated on a relative scale of zero to four, with maximum motility given a rating of four.

RESULTS AND DISCUSSION

These dyes, when used in the concentration range stated above, were relatively nontoxic to spermatozoa in all diluters tested, as evaluated by sperm motility. As shown in Table 2, there was no reduction in per cent motile sperm or rate of motility after incubation for 15 min. at 37° C. with either coriphosphin or

TABLE 2

Effect of coriphosphin and acridine orange on the livability of spermatozoa in various diluents

(Mean of ten samples)

Diluent	Storage time at 5° C.	Control		Coriphosphin						Acridine Orange					
				1:10,000		1:25,000		1:40,000		1:10,000		1:25,000		1:40,000	
		Motility		Motility		Motility		Motility		Motility		Motility		Motility	
		%	Rate	%	Rate	%	Rate	%	Rate	%	Rate	%	Rate	%	Rate
	(hr.)														
E.Y.C.[a]	Initial	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6
	12	59	2.2	53	1.8	54	2.0	56	2.1	56	2.1	57	2.2	57	2.1
	24	55	2.0	47	1.6	49	1.6	50	1.7	49	1.8	50	1.8	50	1.8
E.Y.P.[b]	Initial	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6
	12	57	2.1	53	1.7	54	1.8	55	2.0	54	1.8	55	2.0	53	2.0
	24	51	1.8	44	1.4	45	1.6	47	1.6	42	1.6	46	1.6	46	1.6
Whole milk	Initial	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6
	12	58	2.2	49	1.8	53	2.0	57	2.1	53	2.0	53	2.0	53	2.0
	24	54	2.0	43	1.6	47	1.6	48	1.8	44	1.7	46	1.7	47	1.6
70% W.M.[c]	Initial	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6	63	2.6
+	12	57	2.2	50	1.8	53	2.0	57	2.0	53	2.0	56	2.0	53	2.0
30% E.Y.C.[a]	24	54	2.0	43	1.4	47	1.6	50	1.6	47	1.8	49	1.7	49	1.6

[a] One part egg yolk and one part 2.9% sodium citrate dihydrate.

[b] One part egg yolk and four parts of the following: 2.0 g. Na_2HPO_4 + 0.2 g. KH_2PO_4 in 100 cc. H_2O.

[c] Homogenized whole milk.

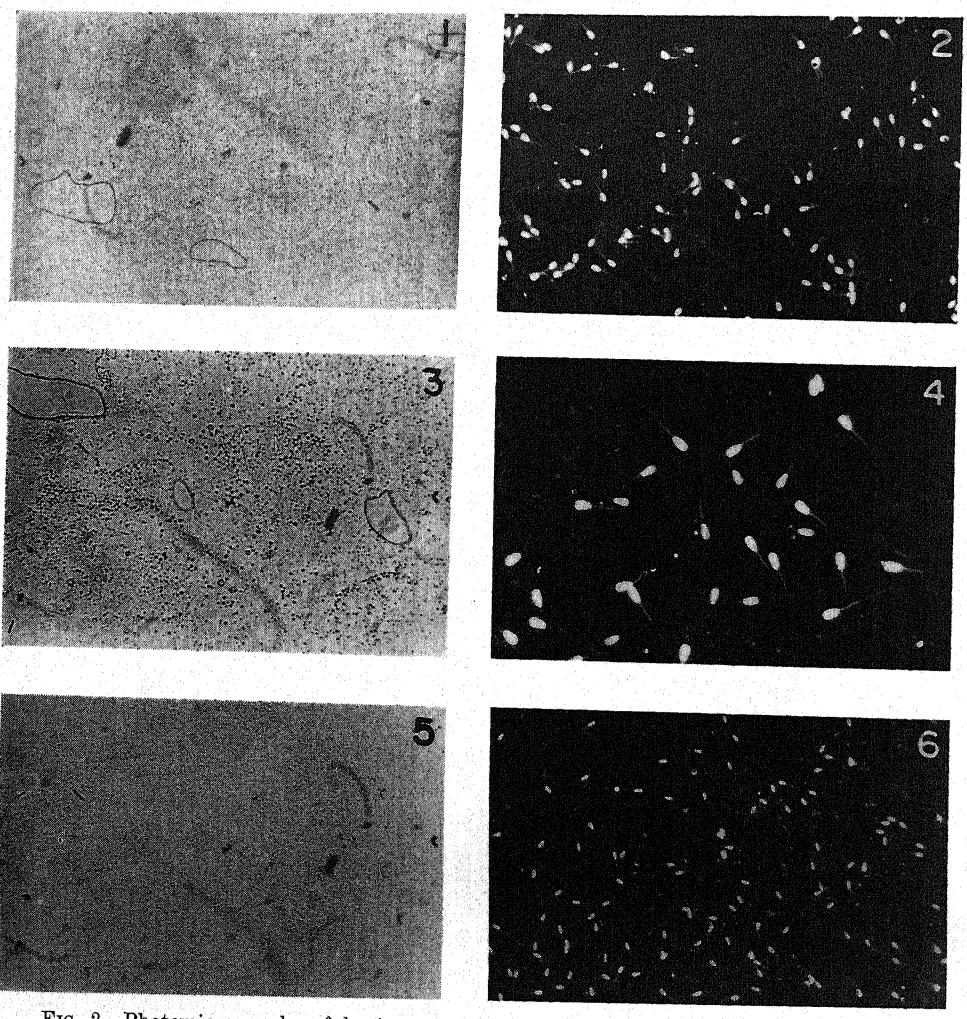


FIG. 2. Photomicrographs of bovine spermatozoa stained with fluorochromes and illuminated with either white or ultraviolet light. The pictures on the left were taken in white light; those on the right are the same fields in ultraviolet light.

(1 and 2) Sperm in whole milk diluter, magnification 133 ×.
(3 and 4) Sperm in whole milk diluter, magnification 287 ×.
(5 and 6) Sperm in yolk-phosphate, magnification 133 ×.

acridine orange when compared with the control, unstained sample. Stained samples which were stored at 5° C. for 12 to 24 hr. exhibited only a slight reduction in motility compared with control samples stored for a comparable period. These results show that these fluorescent dyes cause little or no reduction in the motility of bovine spermatozoa when used in the low concentration tested. This concurs with the findings of Strugger and Rosenberger (6), that acridine orange was relatively nontoxic to goat sperm in a glucose-phosphate diluent. However, the slight toxicity of the fluorochromes to sperm during storage indi-

cates that the addition of the dyes should be delayed until a short time before examination.

Spermatozoa stained with fluorescent dyes and observed in ultraviolet light were very clearly visible even in such opaque media as whole milk and yolk-phosphate diluters. There was little interference from fat globules or other extraneous material, and evaluation of sperm motility could be made readily. Figure 2 illustrates the outstanding visibility of the fluorescing sperm observed in ultraviolet light, as compared to the same samples observed in conventional white light.

The aforementioned simple staining procedure could be easily adopted in any research or field laboratory for the observation of sperm motility where opaque diluents are being used. With only a small addition to the equipment commonly found in artificial insemination laboratories, the sperm can be made more visible by fluorescent staining, resulting in greater accuracy in the estimation of the motility and per cent motile sperm in a sample of semen. Thus, one of the difficulties encountered in the use of opaque media such as whole milk and yolk-phosphate diluters can be easily overcome.

Staining of sperm with fluorochromes has already greatly facilitated the study of sperm morphology (1, 2, 3, 5). If fertility is unaffected, as shown by Strugger and Rosenberger (6), this research tool also can be utilized in other studies involving sperm, where greater visibility of the spermatozoa is desirable, such as: studies of the spermatozoa in the female tract, studies on the mechanics of fertilization, location of the fertilized egg in oviduct effluent, etc. Other applications of this staining procedure also may be found.

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INFLUENCE OF SPERM CONCENTRATION, INITIAL FRUCTOSE LEVEL, AND INITIAL PER CENT MOTILITY ON THE FRUCTOLYTIC ACTIVITY OF BOVINE SEMEN [1]

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SUMMARY

Three semen characteristics—sperm concentration, initial fructose level, and initial per cent motility—were measured in 262 semen specimens collected by electroejaculation from ten Hereford bulls, and fructose utilizations were determined after 20, 40 and 60 min. of incubation at 37° C. The zero-, first- and second-order partial and multiple correlations were calculated from the data on total, among-bulls, and ejaculates-within-bulls bases. Each of the three semen characteristics, independent of the other two, was correlated ($P < 0.01$) with fructolysis on within-bulls bases. Multiple correlations of the three semen characteristics with fructolysis were significant ($P < 0.01$) on within-bulls bases. Multiple covariance analysis showed no significant among-bulls differences in fructolysis, indicating that, in this system, differences in fructose utilization may be accounted for by differences in sperm concentration, initial fructose level, and initial per cent motility.

The influence of sperm concentration on various measures of bull semen fructolysis has been recognized for some time (1, 2, 3, 10) and an influence of sperm motility has been claimed (1, 2) but not supported by published statistical analyses of the data. Recently, the important role of the initial fructose level has been established and it has been demonstrated that the fructolytic rate of this system is substrate-limited (5, 6, 7, 13). The role of the initial substrate level in sperm metabolism has since been confirmed in studies on human semen (4, 11), although no influence of motility, independent of sperm concentration and initial fructose level, on fructolysis was found.

Most of the modern work reported on bull semen metabolism has been based on ejaculates from dairy bulls in artificial insemination (A.I.) studs. These dairy bulls are selected for good semen quality, i.e., high sperm concentration and high initial per cent motility and the means reported in the literature for these characteristics have been high (6, 7, 10). It has been the experience of these investigators that very few poor semen quality specimens, i.e., ejaculates with less than 0.500 billion sperm per milliliter and less than 50% initial motility become available for study from bulls in an A.I. stud. It would be expensive to obtain and raise a group of unselected dairy bull calves, in order to have a population of bulls producing specimens with a greater range of variability in semen quality, although the advantage of such material is apparent.

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The opportunity arose, during the course of another study, to examine the factors influencing the fructolytic activity of semen specimens from a group of Hereford bulls, unselected for semen quality. These specimens were found to demonstrate great variability in characteristics and included many semen specimens of poor quality.

MATERIALS AND METHODS

The semen specimens used in this study were collected by electroejaculation (14) from ten sexually mature Hereford bulls, maintained on native pasture during the months of June and July, 1958 at the UT-AEC Agricultural Research Laboratory, Oak Ridge, Tennessee. Two consecutive semen samples of about 5 ml. each were collected from each of the bulls on Monday, Wednesday, and Friday for a total of six specimens per bull per week.

The specimens were handled, diluted, incubated, and prepared for fructose assay as previously described (6). Fructose utilizations were determined after 20, 40, and 60 min. of incubation at 37° C., using the method of Roe (15). Statistical analyses of the data followed the methods outlined by Snedecor (16).

RESULTS AND DISCUSSION

Mean sperm concentration for the 262 semen samples was 0.238 billion per milliliter, mean initial fructose level was 2.58 mg. per milliliter, and mean initial motility was 53%. Mean fructose utilizations per milliliter of semen were 0.26 mg. in 20 min., 0.43 mg. in 40 min., and 0.55 mg. in 60 min. at 37° C.

Upon first consideration, metabolic studies on semen collected by electroejaculation might seem to present some rather special problems. However, no differences have been reported for the semen characteristics (9) or for the fertility (12) of ejaculates collected by electroejaculation, as compared to ejaculates collected from the same bulls by artificial vagina. The high frequency of collection (six ejaculates per week) might have had some influence on the semen characteristics, although there is no indication from these data that this occurred during the relatively short course of the study.

The zero-, first- and second-order partial and multiple correlations calculated from the data on total, among-bulls, and ejaculates-within-bulls bases are presented (Table 1). The lack of correlation between sperm concentration and initial

TABLE 1

Correlations among various semen characteristics (continued)

Correlated factors	Coefficients of correlation and degrees of freedom		
	Total	Among bulls	Ejaculates within bulls
Zero-order correlations—degrees of freedom	260	8	251
Sperm concentration versus			
initial fructose level	0.02	0.08	−0.01
initial motility	0.38[b]	0.63[a]	0.28[b]
20-min. utilization	0.49[b]	0.68[a]	0.46[b]
40-min. utilization	0.51[b]	0.65[a]	0.47[b]
60-min. utilization	0.49[b]	0.62	0.44[b]

TABLE 1

Correlations among various semen characteristics (concluded)

Correlated factors	Coefficients of correlation and degrees of freedom		
	Total	Among bulls	Ejaculates within bulls
Initial fructose level versus			
initial motility	0.28[b]	0.46	0.18[b]
20-min. utilization	0.44[b]	0.63[a]	0.42[b]
40-min. utilization	0.50[b]	0.71[a]	0.44[b]
60-min. utilization	0.53[b]	0.72[a]	0.46[b]
Initial motility versus			
20-min. utilization	0.42[b]	0.90[b]	0.31[b]
40-min. utilization	0.46[b]	0.87[b]	0.34[b]
60-min. utilization	0.47[b]	0.85[b]	0.35[b]
First-order partial correlations—degrees of freedom	259	7	250
Independent of fructose level, sperm conc. versus			
initial motility	0.39[b]	0.67[a]	0.29[b]
20-min. utilization	0.54[b]	0.82[b]	0.51[b]
40-min. utilization	0.57[b]	0.85[a]	0.53[b]
60-min. utilization	0.56[b]	0.82[a]	0.50[b]
Independent of motility, sperm conc. versus			
initial fructose level	−0.09	−0.31	0.07
20-min. utilization	0.40[b]	0.33	0.40[b]
40-min. utilization	0.41[b]	0.27	0.41[b]
60-min. utilization	0.38[b]	0.21	0.38[b]
Independent of sperm conc., fructose level versus			
initial motility	0.29[b]	0.53	0.19[b]
20-min. utilization	0.50[b]	0.79[a]	0.47[b]
40-min. utilization	0.57[b]	0.88[b]	0.50[b]
60-min. utilization	0.59[b]	0.86[b]	0.52[b]
Independent of motility, fructose level versus			
20-min. utilization	0.38[b]	0.57	0.38[b]
40-min. utilization	0.44[b]	0.72[a]	0.41[b]
60-min. utilization	0.47[b]	0.71[a]	0.43[b]
Independent of sperm conc., motility versus			
20-min. utilization	0.29[b]	0.83[b]	0.21[b]
40-min. utilization	0.33[b]	0.78[a]	0.24[b]
60-min. utilization	0.36[b]	0.76[a]	0.26[b]
Independent of fructose level, motility versus			
20-min. utilization	0.35[b]	0.88[b]	0.26[b]
40-min. utilization	0.38[b]	0.87[b]	0.29[b]
60-min. utilization	0.40[b]	0.85[b]	0.31[b]
Second-order partial correlations—degrees of freedom	258	6	249
Independent of fructose level and motility, sperm concentration versus			
20-min. utilization	0.47[b]	0.64	0.46[b]
40-min. utilization	0.50[b]	0.74[a]	0.48[b]
60-min. utilization	0.48[b]	0.64	0.46[b]
Independent of sperm concentration and motility, fructose level versus			
20-min. utilization	0.45[b]	0.75[a]	0.45[b]
40-min. utilization	0.52[b]	0.87[b]	0.48[b]
60-min. utilization	0.54[b]	0.83[b]	0.49[b]
Independent of sperm concentration and fructose level, motility versus			
20-min. utilization	0.18[b]	0.79[a]	0.14[a]
40-min. utilization	0.21[b]	0.77[a]	0.17[b]
60-min. utilization	0.24[b]	0.70	0.20[b]
Multiple correlations—degrees of freedom	258	6	249
Sperm concentration, fructose level, and motility with			
20-min. utilization	0.67[b]	0.96[b]	0.63[b]
40-min. utilization	0.72[b]	0.97[b]	0.66[b]
60-min. utilization	0.73[b]	0.96[b]	0.67[b]

[a] Statistically significant ($P < 0.05$).
[b] Statistically significant ($P < 0.01$).

fructose level, on both zero- and first-order partial bases, confirms a previous study with A.I. bulls (6), which showed that these variables are independent of each other. The correlation ($P < 0.01$) between sperm concentration and initial motility, on both zero- and first-order partial bases, indicates that those samples with the higher sperm concentrations tend to have the higher motilities and demonstrates the need for an evaluation of the influence on fructolysis of each of these two variables, independent of the other. The correlation among the three variables must be recognized, and second-order partial correlations calculated, to account for the influence on fructolysis of each variable, independent of the other two. When this has been done, it becomes apparent (Table 1) that each of the three variables, sperm concentration, initial fructose level, and initial per cent motility, influences the fructolysis of this system. The multiple correlation, 0.66, of 60-min. fructose utilization with sperm concentration, initial fructose level, and initial per cent motility on an ejaculates-within-bulls basis, demonstrates the combined influence of the three variables on fructolysis.

An analysis of covariance was used to test for differences among bull mean fructose utilizations adjusted to average sperm concentration, initial fructose level, and initial per cent motility. There was no significant difference among bull means in fructose utilization when mean squares free of regression effects were tested (Table 2). This indicates that the fructolytic activity of this system is primarily dependent on sperm concentration, fructose level, and motility, and confirms previous work with A.I. bulls, which concluded that the seminal plasma, apart from its fructose content, had no measurable influence on fructolysis (7). This, however, is not in agreement with reports on fructolysis in bull semen (8) and in human semen (4, 11), where the seminal plasma was found to exert a marked influence on fructolysis independent of its fructose content.

TABLE 2

Analysis of covariance of fructose utilizations. Deviations from regression

Source of variation	Degrees of freedom	Mean square: Fructose utilized in: 20 min.	40 min.	60 min.
Among bulls	9	612	864	1,536
Ejaculates within bulls	249	322	636	922
Total	258			

GENERAL DISCUSSION

The factors influencing the fructolytic activity of bovine semen have been studied and the papers reporting the influence of sperm concentration (1, 2, 3, 10) and of initial fructose level (5, 6, 7) have been confirmed and, in addition, the influence of initial per cent motility has been demonstrated. It is evident that any study which attempts to relate other semen attributes, e.g., fertility, to measures of bovine semen fructolysis must either account and adjust for the influence of sperm concentration, fructose level, and motility or use a measure of fructolysis which has been demonstrated to be independent of all three variables.

Also, it might be suggested that sperm concentration and fructose level are not semen sample quality factors per se, whereas motility is and, therefore, that a more suitable approach to fertility studies might include an adjustment of fructolysis for the influence of sperm concentration and fructose level and then the calculation of the multiple correlation of fertility with adjusted fructose utilization and motility.

The absence of bull differences in fructolytic activity, independent of sperm concentration, fructose level, and motility effects, in this study and in another study with A.I. bulls (6), leads to the conclusion that differences in fructolytic activity may not be useful in predicting differences in other bovine semen attributes.

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METABOLISM OF BOVINE SEMEN. VIII. FLUOROACETATE AS AN INHIBITOR OF SPERMATOZOAN MOTILITY, GLYCOLYSIS, AND RESPIRATION[1, 2]

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SUMMARY

Sodium fluoroacetate in concentrations as high as 0.01 *M* has been tested as an inhibitor of the motility, glycolysis, and respiration of bovine spermatozoa in heated skimmilk, egg yolk–citrate (1:3), and modified Ringer solution. Effective inhibition of motility and glycolysis was not obtained. Endogenous respiration was 70% inhibited by 0.01 *M* fluoroacetate; respiration with substrates of 0.01 *M* acetate, glucose, and fructose was inhibited 70–90%. Production of $C^{14}O_2$ from acetate-1-C^{14} was almost completely blocked by fluoroacetate.

Metabolic inhibitors have received consideration as a means of prolonging the life of spermatozoa (6, 7, 8, 10, 12, 13, 21). Although many factors are involved in determining the effectiveness of inhibition of spermatozoan metabolism (2, 3, 14, 19, 20), reversible inactivation has been demonstrated (7, 8, 10, 18).

Fluoroacetate is known to inhibit aconitase (4) and, thus, is an effective inhibitor of acetate metabolism. Because of the presence of acetate in freshly ejaculated semen (5), and the utilization of acetate by spermatozoa (9), a study of the effects of fluoroacetate on the motility, glycolysis, and respiration of bovine spermatozoa was undertaken and the results are presented in this report.

Experimental Procedure

The following diluents were used in these trials: (a) skimmilk heated to 92° C. for 10 min. (15), (b) egg yolk–2.9% sodium citrate dihydrate (1:3), and (c) modified Ringer solution consisting of 100 ml. of 0.9% NaCl, 4 ml. of 1.15% KCl, 1 ml. of 2.11% KH_2PO_4, and 1 ml. of 3.82% $MgSO_4 \cdot 7H_2O$. Glucose or fructose was added to the Ringer solution to a final concentration of 0.01 *M*. All diluents were adjusted to a pH of 6.6–6.8. Semen samples were collected with the artificial vagina and subjected to routine laboratory evaluation tests.

For the motility trials, the semen samples were diluted to a standard concentration of 15×10^6 cells per milliliter in each diluent and stored at 5° C.

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[3] Part of the data contained in this paper are from a thesis submitted by the senior author to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Master of Science.

Motility estimations were made at 2–168 hr. after dilution, on a microscope equipped with a thermostage set at 37° C. At 168 hr., reactivation of spermatozoa was attempted by replacing the diluent with fresh diluent containing no inhibitor. Motility was observed at periodic intervals following the reactivation attempt.

Glycolysis was studied by measurement of both fructose disappearance and lactic acid accumulation during 3 hr. of incubation at 37° C. Individual semen samples were diluted to a standard concentration of 50×10^6 cells per milliliter. Aliquots for chemical analysis were removed after 0 and 3 hr. of incubation and dispersed immediately in 10% trichloroacetic acid. Filtrates were subsequently analyzed for fructose (11) and lactic acid (1).

Respiration studies were conducted with a Warburg constant volume respirometer. Measurements of oxygen uptake (17) were made with pooled ejaculates from at least three bulls. The ejaculates were mixed and the spermatozoa were washed three times in the centrifuge, then suspended in the appropriate diluent. Spermatozoan concentration was standardized at 500×10^6 cells per milliliter.

Results and Discussion

Studies of the inhibition of motility by the various concentrations of sodium fluoroacetate in heated skimmilk involved eight ejaculates; an additional eight ejaculates were used in testing inhibition in egg yolk–citrate. As shown in Table 1, fluoroacetate in concentrations as high as 0.01 *M* did not effectively inhibit spermatozoan motility. Compared to the control (no fluoroacetate), maximum inhibition was less than 10% in skimmilk and about 30% in egg yolk–citrate. When reactivation of spermatozoa was attempted at 168 hr., no improvement in motility was observed.

Results of measurements of glycolysis are presented in Table 2. The values listed are the means from a trial involving four ejaculates. Similar results were obtained in two other trials involving four ejaculates each in skimmilk and egg

TABLE 1
Inhibition of motility by sodium fluoroacetate
(Mean of 8 ejaculates)

Fluoroacetate concentration	Hours of storage at 4° C.											
	2	4	6	8	24	36	48	72	96	120	144	168
($\times 10^{-4}M$)	(% *motile spermatozoa*)											
Skimmilk diluent												
0	60	60	59	59	59	59	56	55	51	50	46	40
5	60	60	59	58	58	58	56	52	50	50	44	40
10	60	60	58	58	56	56	56	53	50	49	45	40
50	59	59	56	56	55	55	54	54	47	46	43	39
100	60	60	56	56	55	55	54	51	48	47	43	39
Egg yolk–citrate diluent												
0	60	60	59	59	51	51	48	44	44	40	35	34
5	60	59	59	56	50	45	41	38	36	32	28	28
10	59	57	56	54	47	45	41	36	32	31	30	27
50	58	56	55	54	48	45	40	33	31	29	28	25
100	60	55	51	50	46	44	38	30	30	27	24	24

TABLE 2
Inhibition of glycolysis by sodium fluoroacetate

Fluoroacetate concentration	Diluent					
	Skimmilk		Egg yolk–citrate		Ringer-fructose	
	Fructose loss	Lactate gain	Fructose loss	Lactate gain	Fructose loss	Lactate gain
(× 10^{-4} M)	*(mg/hr/10^9 spermatozoa)*					
0	1.30	2.62	0.63	3.56	1.05	0.52
5	1.45	2.28	0.57	2.96	0.71	0.52
10	1.47	2.36	0.55	2.85	0.65	0.38
50	1.06	2.05	0.40	2.61	0.53	0.43
100	1.18	2.06	0.43	2.56	0.67	0.44

yolk–citrate and 14 ejaculates in Ringer-fructose. Although there is a tendency toward lower values in fluoroacetate-containing samples than in controls, the variation was such that any true inhibition of glycolysis, if present, is of little consequence.

The failure of fluoroacetate to satisfactorily inhibit motility and glycolysis essentially eliminates its consideration for use in the preservation of semen; conservation of energy in these cells almost certainly must involve a reduction in glycolysis, the principal energy source.

In contrast to the lack of inhibition of motility and glycolysis, respiration was markedly inhibited by fluoroacetate (Table 3). Oxygen uptake by spermatozoa was reduced in each of the three diluents by all concentrations of fluoroacetate studied. Direct comparisons among diluents are not valid, inasmuch as different semen samples were used in each of the diluents. It appears, how-

TABLE 3
Inhibition of respiration by sodium fluoroacetate

Fluoroacetate concentration	Minutes of incubation at 37° C.						
	15	30	45	60	90	120	180
(× 10^{-4} M)	*(mean microliters O_2 uptake/10^9 spermatozoa)*						
	Skimmilk diluent						
0	67	153	222	283	392	464	601
5	55	118	164	203	279	347	474
10	55	118	164	203	278	345	466
50	39	84	120	154	214	274	388
100	35	40	115	144	197	252	341
	Egg yolk–citrate diluent						
0	95	177	263	350	482	578	633
5	72	138	198	263	355	492	586
10	72	134	196	249	338	464	561
50	72	122	182	229	319	440	554
100	71	116	178	217	293	365	461
	Ringer-glucose diluent						
0	99	186	280	367	455	552	612
5	83	184	270	355	455	545	609
10	83	175	256	317	418	539	572
50	80	153	216	273	356	447	527
100	55	106	137	186	230	293	366

ever, that less inhibition was obtained in egg yolk–citrate than in skimmilk or Ringer-glucose. Tosic and Walton (16) observed that egg yolk increased the respiration of spermatozoa, and found this enhancement of respiration associated with the fat-free protein residue of egg yolk. Possibly the same or a similar factor may account for the resistance to fluoroacetate inhibition found in egg yolk–citrate.

Additional respiration studies were conducted in Ringer solution, to determine the effect of substrate on the extent of inhibition. These studies are summarized in Table 4. The substrate in each instance was present at a concentration of 0.01 *M*. Inhibition was determined by comparing the oxygen uptake by washed spermatozoa in 0.01 *M* fluoroacetate to that with no fluoroacetate.

Oxygen uptake from acetate was inhibited to a greater extent than that from glucose or fructose, whereas endogenous respiration was less effectively inhibited. It appears that the endogenous respiration which was not inhibited by fluoroacetate might account for the oxygen uptake observed in fluoroacetate-inhibited spermatozoa in acetate substrate. This assumption was tested by measuring $C^{14}O_2$ production from acetate-1-C^{14} in the inhibited system; under these conditions practically no $C^{14}O_2$ was obtained. Elliott and Kalnitsky (4) reported that fluoroacetate inhibited aconitase, and this blockage obviously eliminates acetate oxidation by spermatozoa.

The results presented indicate that acetate, although readily metabolized by spermatozoa, is not a critical metabolite in a metabolic pathway essential to the maintenance of motility. Inhibition of respiration was observed, regardless of whether acetate, glucose, or fructose was the substrate. In all probability, inhibition also would be observed with lactic acid as substrate. The disappearance of fructose and accumulation of lactic acid at normal rates in inhibited samples indicates that blockage of respiration does not interfere with the disposition of lactic acid formed from fructose.

TABLE 4

Effect of substrate on inhibition of respiration by fluoroacetate

Substrate added	Hours of incubation at 37° C.		
	1	2	3
(0.01 M)	*(% inhibition)*		
Experiment 1 (mean of 3 trials)			
Acetate	90[a] ± 3[b]	89 ± 2	89 ± 2
Glucose	73 ± 3	79 ± 2	83 ± 1
Experiment 2 (mean of 3 trials)			
Acetate	86 ± 2	86 ± 3	88 ± 2
Fructose	73 ± 3	79 ± 1	83 ± 1
Experiment 3 (mean of 4 trials)			
None (endogenous)	69 ±10	72 ± 9	72 ± 9
Acetate	88 ± 3	87 ± 2	88 ± 2
Acetate-1-C^{14}			99.2[c] ± 0.2

[a] Of O_2 uptake.
[b] Standard error of the mean.
[c] Of $C^{14}O_2$ production.

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A SEXUAL DIMORPHISM IN THREE SOMATIC TISSUES OF CATTLE

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SUMMARY

A method of illustrating a sexual dimorphism in three somatic tissues of cattle has been perfected. A small, mildly chromotropic, planoconvex chromatin mass found predominantly in females was interpreted to be the sex chromatin reported present in many other mammalian species. In liver, pancreas, and adrenal the sex chromatin appeared in 68, 62, and 61%, respectively, of the cells examined, but in only 10% or less of the male cells. The possible uses of sexual dimorphism at the cellular level in elucidating embryonal development, and its proposed use as a method of antenatal sex determination in veterinary practice, were considered. Unknown tissues were properly classified as to sex in 19 of 20 cases.

Barr and Bertram (1) established a sex difference in mammalian somatic cells based on the presence of a small intranuclear chromatin mass in the female cell. A similar chromatin clump was seldom found in male cells. Since their initial observation, a sexual dimorphism has been reported in many tissues of human and other mammalian species.

Moore and Barr (9) provided the first evidence that a sex difference existed in somatic tissues of Artiodactyla, by describing the sex chromatin in neural nuclei of the deer and goat. Later, Moore *et al.* (11) reported similar findings for the nervous tissue of cattle, but the presence of heavily staining karyosomes obscured any sex-specific differences in other somatic tissues.

A study was initiated in 1957 to investigate the possibility of a sexual dimorphism in somatic tissues of cattle. During the exploratory work, it became evident that the sex chromatin, if present, could not be delineated from the multiple coarse chromatin clumps inherent in many somatic cells of cattle. An effort was made to remove the extra-karyosomal material from the nuclei of these cells. A detailed description of the methods employed and the results follows.

EXPERIMENTAL PROCEDURE

For the demonstration of the sex chromatin in cattle, small samples of liver were collected at slaughter from ten Holstein cows, five Holstein bulls, and five Hereford steers. Pancreas and adrenal tissue samples were taken from another two Holstein cows and an aged Holstein bull.

The tissues were immediately removed to modified Davidson's fixative for 24 hr. Modified Davidson's solution comprises 30 parts 95% ethyl alcohol, 20 parts glacial acetic acid, ten parts formalin (37%), and 20 parts distilled water.

Following fixation, the tissue samples were trimmed and placed in 70% alcohol for 1 hr. The sections were progressively dehydrated in 80%, 95%, and absolute alcohol for periods of 1 hr. each, previous to embedding in paraffin. Once

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the blocks of paraffin had solidified, the sections were cut at 10 μ and attached to glass slides by gentle warming.

Prepared slides were placed in xylene for 3 min. to remove the paraffin and then transferred to a 1.5% celloidin solution. Celloidin, a purified form of the nitrocellulose collodion, was required to protect the tissues against the stringent acid hydrolysis which followed.

After 4 min. in celloidin, the slides were briefly air-dried until the protective film became firm, and were finally hardened in 70% alcohol. Thus protected, the slides were transferred to 7 *N* HCl for 15 min., to undergo a period of hydrolysis. Upon completion of the hydrolysis and prior to entering the buffered staining solution, it was essential to carefully rinse the slides in distilled water, to prevent the alteration in pH of the stain by acid carry-over.

The stain used was a Thionin solution buffered to pH 5.7 $\pm$ 0.2. The saturated solution of Thionin stain in 50% alcohol was adjusted to the desired pH with Michaelis' universal buffer and 0.1 *N* HCl, according to the formula presented by Gortner and Gortner (2).

After 15 min. had elapsed, the slides were removed from the stain, briefly differentiated in 70% alcohol to remove the excess dye, then dehydrated for 4 min. in reagent-grade *p*-dioxane. Final clearing in xylene prior to mounting with Diaphane completed the staining procedure.

Slides were examined under oil immersion and, using a 95 $\times$ objective and 20 $\times$ eyepieces, it was possible to obtain a 1,900 $\times$ magnification. Resolution at this magnification was good.

A slide from each liver sample of the ten females and ten males was stained and a random number was attached to this slide by a disinterested person. With the sex unknown to the observer, the number of cells containing a definable chromatin clump attached to the nuclear membrane was recorded. The sexes of the animals were predicted from this information. The observations were included only in instances where the nuclear membrane was clearly defined and the cell appeared normal. One hundred cells from each slide were examined.

The procedure established for demonstrating a sex difference in the liver cells of cattle was tried on pancreas and adrenal tissues from two cows and a bull, so as to evaluate its application to other somatic tissues. Cell counts and general observations were recorded.

Photographs depicting the typical appearance of liver tissue for each sex were taken at 960 $\times$ magnification and enlarged to 2,000 $\times$ the original cell size.

RESULTS

The nuclei of the liver, pancreas, and adrenal emerged from the rigorous acid hydrolysis and staining procedure detailing the nuclear membrane and several intranuclear inclusions. The plasmosome which appeared inconstant in shape and inconsistent in its uptake of stain was generally present, accompanied by a number of stained chromatin clumps. One of these clumps, a small, sharply defined planoconvex, slightly chromotropic mass, apposed to the inner surface of the nuclear membrane, was evident in many of the tissues derived from females.

A similar-sized chromatin knot, exhibiting the same position and color characteristics, was seldom present in cells of male origin.

It was logical to interpret that the chromatin mass discernible in the cells of the three tissues, but predominant in the one sex, corresponded to the sex chromatin reported in the neural nucleus of this and a number of other mammalian species.

Generally, the nuclei of stained cells were vacuolar in appearance and, in many cases, cells near the surface of the sections were almost devoid of any chromatin material. Those toward the center of the section responded in a more consistent manner to acid and stain. In the male cells that had undergone an almost complete removal of karyosomal material, a small triangular, sharply staining body was found attached to the nuclear membrane. Its size was approximately half that of the sex chromatin found in female tissues.

A total of 68% of the female liver cells examined contained the sex chromatin, whereas only 10% of the male cells retained any suggestion of its presence. No obvious differences were noted between castrate and noncastrate male cells. The incidence of the sex chromatin in female pancreatic cells approximated 62%, whereas similar cells of males contained only 10%. In comparison, the sex chromatin was found in 61% of female adrenal cells examined, but could be recorded in only 9% of the male nuclei. These figures are summarized in Table 1.

TABLE 1

The incidence of the sex chromatin in three somatic tissues of cattle listed according to sex

Type of tissue	Sex of tissue	Incidence of sex chromatin
		(%)
Liver	Female	68
	Male	10
Pancreas	Female	62
	Male	10
Adrenal	Female	61
	Male	9

Photographs of the liver cells contrast the typical sex difference, dependent on the presence of the small overt chromatin dot (Figures 1 and 2). It must be remembered, however, that the photographs are unable to adequately relate the chromotropic properties of the sex chromatin. It should also be recognized that the inability to maintain a focus for each cell in the photographic field often exaggerates the significance of other karyosomal particles. Under the microscope these particles resolve into shells of chromatin, whereas the sex chromatin, by virtue of its dense heterochromatic mass, remains visible over a larger range of foci.

To validate the staining technique as a method for sorting tissues according to sex difference, 20 stained slides, one taken from each liver sample, were randomly numbered by a disinterested person. Observations entailing the number of sex chromatin per tissue group were compiled. This information was utilized to predict the sex of tissue and, hence, the sex of the animal.

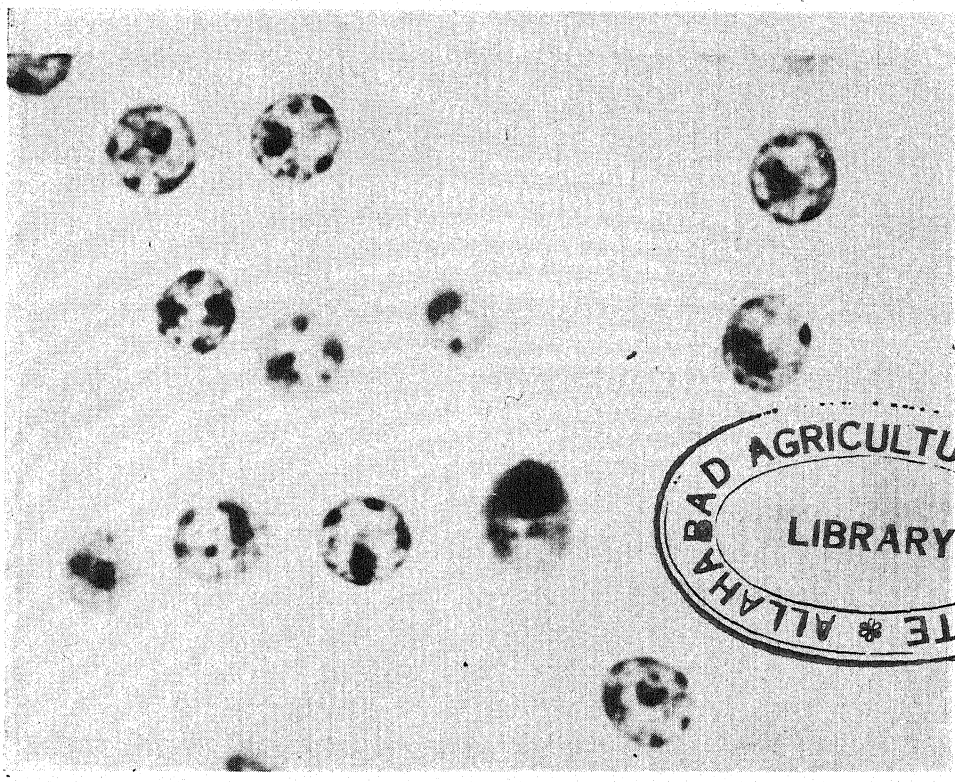


FIG. 1. Liver nuclei of female origin. The tissue was stained under standard conditions with other cell types and displays the sex-specific sex chromatin. The two nuclei, upper right, clearly illustrate the chromatin dot attaced to the inner face of the nuclear membrane. (2,000 ×)

The results of these predictions are included in Table 2. The sex of 19 tissues was correctly predicted, but the remaining slide, random number 709, was mistakenly presaged as a female. Poor stain uptake and the large number of karyosomal particles contributed to the error.

DISCUSSION AND CONCLUSIONS

The sex chromatin, discovered by Barr and Bertram (1) in cat neural cells, and which is thought to be a derivative of the XX sex chromosome complex, is clearly perceptible in liver, pancreas, and adrenal tissues of cows. The inability of Moore *et al.* (11) to show a sexual dimorphism in other than neural tissue was undoubtedly dictated by the methods which they employed. They stained their 16 somatic tissue samples with Harris' haemotoxylin and eosin and Feulgen's stain, and were unable to differentiate the sex chromatin from a large group of well-stained karyosomes. Feulgen's method employs a short period of acid hydrolysis which is not severe enough to remove the extra chromatin clumps. Klinger (6) adapted Feulgen's method, suggesting that 5 *N* HCl for 20 min. was the most satisfactory period of hydrolysis for clearing human tissue.

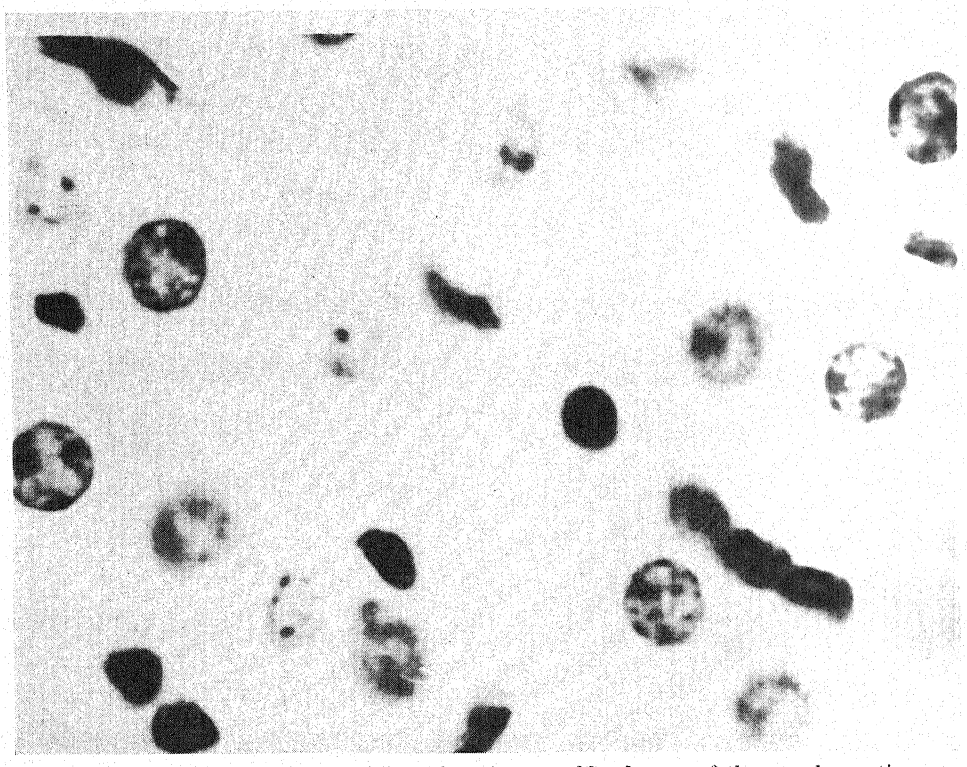


FIG. 2. Liver nuclei of male origin. There is a notable absence of the sex chromatin, or a similar body, in nearly all of these cells. (2,000 ×)

TABLE 2

Prediction of the sex of liver tissues according to the nuclear morphology

Random number of slide	Per cent of nuclei including the sex chromatin	Predicted sex	Actual sex
32	68	Female	Female
709	45	Female	Male
960	66	Female	Female
5x	76	Female	Female
75	72	Female	Female
K	7	Male	Male
371	7	Male	Male
401*	16	Male	Male
40x	84	Female	Female
44*	6	Male	Male
83*	10	Male	Male
56	45	Female	Female
70	65	Female	Female
109*	13	Male	Male
3x	16	Male	Male
739	61	Female	Female
38	75	Female	Female
1*	10	Male	Male
301	70	Female	Female
72	8	Male	Male

* Castrate males.

By further increasing the acid concentration to 7 *N* HCl and reducing the immersion period to 15 min., a satisfactory method has been evolved to remove excess chromatin from cattle somatic cells. As Klinger (6) points out, after intense acid hydrolysis of the nuclei, the sex chromatin remains merely a cell marker, and is of little use in providing data on the size, ultrastructure, and origin of the nuclear inclusion.

Graham and Barr (4), working with the cat, and Walsh (14) with the golden hamster, had difficulty in identifying the sex chromatin in liver tissue, but Prince *et al.* (12) and Moore and Barr (10), working with the monkey and human, respectively, did not encounter any similar problems. There was little difference between the staining properties of the three somatic tissues of cattle. It is difficult to resolve the anomalies of the staining characteristics found for different species and cell types.

The initial attempts to identify the sex chromatin in cattle somatic cells were unyielding, as large numbers of grossly staining karyosomes were generally present. An exacting approach to the clearing of the cell with acid and a different method of de-staining to that used by Klinger and Ludwig, eventually led to the "unmasking" of the sex chromatin.

Klinger and Ludwig (7) differentiated stained human tissues in 50 and 70% alcohols, and then dehydrated gradually with 80%, 95%, and absolute alcohol. Resulting from a persistent inability of the cattle cells to retain the stain against the effects of alcohol, it was decided to differentiate to a desired color intensity with 70% alcohol, then use *p*-dioxine as the dehydrant. Dioxine only combines with the excess water molecules, and is less likely than alcohol to interact with the nucleoproteins.

Grumbach and Barr (5), in their review of the sex chromatin, are reserved in their opinion as to the apparent absence of the sex chromatin in nonnervous tissues of cattle. The chromosome number of cattle observed by Makino (8) was 60, and included an XY complex for the male. This information, along with the circumstantial evidence involving the XX-chromosome complex with the sex chromatin, made it untenable to accept the premise that the chromatin body was specific only to nervous tissue. However, it is now proven that the sex chromatin is present in other somatic tissue of cattle.

It is of interest to comment on the presence of the small chromatin dot which remains in the nuclear periphery of male cattle cells after extreme hydrolysis. Graham (3) has shown the presence of a body similar to the sex chromatin, but about half the size, in male opossum cells. As the X-chromosome is unduplicated in the male, it is surmised that the X-chromosomal heterochromatin should be discernible in male cells, but experience indicates that in most species the large number of autosomal chromocentres of similar size precludes the identification of the X-chromosome. It is suggested that the weakly chromotropic mass in the periphery of the male cells of cattle could be the heterochromatic rest of the X-chromosomes.

The finding of the sex chromatin in non-neural somatic cells of cattle further clarifies the picture of a sexual dimorphism in mammals. With a technique

now available for identifying sex at the cell level, a more precise understanding of sexual development and formation of embryonal tissues of Artiodactyla should be possible.

Besides being of academic interest, the detection of sex-dependent differences of nonnervous cattle cells may have some applications in the field. Serr *et al.* (13) proposed that the study of cells in amniotic fluid might be useful in antenatal sex determination in veterinary practice. As Moore *et al.* (11) were unable to detect the sex chromatin in non-neural somatic tissues, they could not accede to this idea. However, with the method described in this paper showing the imprint of sex in readily available cells, it is feasible that antenatal sex determination may be possible. The amniotic sac of cows is accentuated and voluminous, so that fluid containing the exfoliated cells of the offspring might be obtained by aspiration without injuring the foetus.

Another aspect of utilizing a sexual dimorphic cell marker should be explored. If the in utero sex of the embryo can be determined in the cow, it may be possible to gain an understanding of the effects of hormonal imbalances and maternal anomalies on embryo development. Information from these studies may be helpful in elucidating maternal interactions in other mammalian species. Unfortunately, the availability of intersexes of domestic species is limited as farmers generally slaughter these animals. The work of Moore *et al.* (11) showing that the freemartin is chromosomally a female is a good example of the application of somatic cell sex differences to the understanding of a functional aberration. In the future, similar studies may lead to a more complete comprehension of sex differentiation and its physiological ramifications.

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EFFECTS OF VARIOUS POSTPARTUM TREATMENTS UPON THE REPRODUCTIVE EFFICIENCY OF DAIRY COWS[1,2]

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SUMMARY

The effects of postpartum treatments on subsequent reproductive performance were observed on 305 reproductive cycles of 137 Holstein cows from 1953 to 1957.

Cows were assigned to treatment lots, as they freshened, within Group A, normal calving, or Group B, abnormal calving (dystocia and/or placenta retained more than 12 hr.). Animals in Group A, Lot I, received no treatment, while those in Lot II received one intrauterine administration of one oblet containing 500 mg. Aureomycin HCl within one day after parturition. In Group B, the animals usually were treated daily until cured. Lot I received oblets containing 15 g. sulfamethazine (Sulmet) and Lot II received only Aureomycin. A no-treatment lot, Lot III, was added to Group B after the experiment had been in progress about 2 yr. Owing to the few observations and the great variation in the behavior among animals in this lot, the data were evaluated by case studies.

Among Group A cows, no significant differences in breeding efficiency due to treatment could be demonstrated between Lots I and II. Cows in Group B responded more favorably to Aureomycin than to Sulmet treatments (P *ca.* 0.09 for conception interval). The breeding efficiency of cows in Lot B-II approached the level of normal cows. It was shown that some abnormally parturient cows must be treated, to survive.

Parturition and the onset of lactation by themselves represent a period of stress for dairy cows. In addition, many parturitions divert from an accepted normal and desirable pattern. Slight to severe disturbances often accompany them or are followed by conditions which bring about infection, inflammation, and systemic toxicity. Such abnormalities frequently lead to temporary and even to complete sterility. Retained fetal membranes, 8.3 to 12.9% of all parturitions in brucellosis-free herds (7, 8, 11), and dystocia, 13.1% (7) are of prime importance in this respect. They are generally diagnosed without difficulty, although retention of some parts of the placenta and minor cases of dystocia may and will be overlooked even by the attentive herdsman; some of these may cause equally severe disturbances when neglected.

Dystocia occurred most frequently in first-calf heifers and diminished with advancing age (7), whereas retained placenta cases were lowest in first-calf heifers but increased in numbers with advancing age (9, 13). Both have been shown to contribute materially to temporary and permanent sterility (7, 12). To decrease these losses, many kinds of treatments have been tested and recommended in the past. More recently, treatments with a variety of antibiotics have proved to be especially useful in controlling the after-effects of these abnormalities.

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[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, New Brunswick.

[2] This study was part of a Northeast Regional Project (NE-1, Causes and Prevention of Reproductive Failures in Dairy Cattle), a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds of the United States Department of Agriculture.

Wilson (16) found penicillin to be superior to any other available agent at the time of observation for the treatment of metritis. To avoid toxicity generally caused by retained fetal membranes, Newlin (10) reported very favorable results with 0.5 to 1.0 g. intrauterine doses of Terramycin, administered at intervals of 36–48 hr. after calving. Bosshart (2) used larger doses of 1 and 2 g. of Terramycin with about equally good results. Beattie (1) treated 612 retainers with 5-g. oblets of Aureomycin (0.5 g. crystalline Aureomycin hydrochloride) in conjunction with 15–35 g. stilbestrol intramuscular injections. All except two metritic cows overcame the defect without evidence of infection or septicemia. Equally effective results with Aureomycin were obtained by Easterbrooks and Plastridge (5). The retained placenta cases treated at intervals of 48 hr. with 500 mg. of Aureomycin routinely required fewer services per conception, and had a shorter calving interval than either the retained placenta cases treated at 24-hr. intervals or the normal calving group receiving no treatment.

An interest in improving reproductive efficiency in dairy cows prompted this investigation of routine treatments on apparently normally parturient cows and of different treatments on abnormally parturient cows under conditions of practical herd management.

EXPERIMENTAL PROCEDURES

All data for this study were obtained from the records of the Holstein-Friesian herd at the Dairy Research Farm of the New Jersey Agricultural Experiment Station, Sussex. The experiment was started in the fall of 1953 and extended over a period of about 4 yr. During this time, a total of 305 reproductive cycles from 137 cows became available.

All cows were assigned, as they freshened, to one of two groups. Cows which freshened apparently normally were assigned to Group A; those experiencing dystocia and/or retaining their placenta more than 12 hr., were assigned to Group B. Within each group, the cows were alternately assigned, as they calved, to treatment lots in the following manner:

Group A

Lot I—Cows calving apparently normally and receiving no treatment at parturition.

Lot II—Cows calving apparently normally and receiving one intrauterine treatment of Aureomycin (one 500-mg. oblet) within a day after parturition.

Group B

Lot I—Cows having abnormal parturitions and treated with Sulmet (sulfamethazine—15 g. per treatment) until apparently cured.

Lot II—Cows having abnormal parturitions and treated with Aureomycin (500 mg. per treatment) until apparently cured.

Lot III—Cows having abnormal parturitions and not treated, or not treated until death seemed imminent.

It was the responsibility of the herdsmen to assign animals and administer treatments as prescribed. Upon occasion, if an animal did not respond favorably to a particular treatment or if its condition warranted immediate attention, as indicated by a sudden rise in temperature, anorexia, or general toxic symptoms, the animal was removed from the experiment and placed under veterinary care. Those animals, along with a few animals that received, through error, treatments that were not prescribed, comprised a mixed-treatment lot.

Cows experiencing abnormal parturitions (Group B) usually were treated daily either with Sulmet (Lot I) or with Aureomycin (Lot II). On the average, each cow required four treatments in either Lot I or Lot II.

The herdsman, at his discretion, allowed the retained fetal membranes to drop of their own accord, or removed them manually at such a time that they could be removed without observable damage or discomfort to the cow.

Lot III was not added to Group B until about 2 yr. after the start of the experiment. This lot, comprised of cows that experienced abnormal parturitions and received no treatment, was to serve as a control group. On account of the time limitation, only a relatively few records could be accumulated. Since these records were found to be extremely variable, it was decided not to include them in a general analysis of the data but, rather, to present them as individual case studies.

The effects of postpartum treatments were evaluated by several measures; namely, by the number of services per conception, nonreturns to first service, service period (days from calving to conception), and conception interval (days from first service to conception).

The statistical analysis of the data followed standard procedures in respect to the analysis of variance and Chi-square test, as described by Snedecor (14).

RESULTS AND DISCUSSION

Reproduction is seldom extinguished completely, even when conditions are adverse for the best expression of a high level of fertility. Neither is there any clear line of demarcation between impaired fertility and sterility. Because of this limitation, it is often impossible to determine accurately how much any particular factor improves or lowers breeding efficiency. Moreover, a single measure may not give a true indication of the breeding merit of every individual, although some measures may appear to have greater sensitivity than others in detecting differences in breeding efficiency. Consequently, different measures were employed in the evaluation of breeding efficiency.

The summarized data as presented (Table 1) revealed that the breeding efficiency of all cows (232) that conceived again was at a high level. Considering the original 305 reproductive cycles, 13 of these were described in Table 2. The remaining 60 could not be included for various reasons: incomplete records at the close of the experiment, 3; cows not being treated after assignment to a particular lot, 10; cows sold or died before being bred, 19; cows bred but removed before pregnancy could be confirmed, 8; cows sold as trouble breeders, 9; and sterile cows (not in calf after at least 9 mo.), 11.

TABLE 1

Average effects of various postpartum treatments on four criteria of breeding efficiency

	Reproductive cycles	Services per conception	Non-returns to first service	Conception interval[a]	Service period[b]
	(No.)	*(No.)*	*(%)*	*(days)*	*(days)*
Group A—Normal calving; placenta dropped within 12 hr.					
Lot I—No treatment	96	1.47	67	19	97
Lot II—Aureomycin HCl—500 mg.	84	1.78	53	30	102
Group B—Abnormal calvings; dystocia or placenta retained more than 12 hr.					
Lot I—Sulmet (sulfamethazine)—15 g. per treatment until cured					
As planned	18	1.78	50	36	113
Aureomycin after 4+ days[c]	6	2.50	33	62	134
All cows	24	1.96	46	42	118
Lot II—Aureomycin—500 mg. per treatment until cured					
As planned	25	1.64	61	21	96
Plus Sulmet by error	3	1.33	67	6	76
All cows	28	1.61	61	19	94
Grand total—all animals	232	1.65	59	25	101
Mixed animal deleted	223	1.63	59	25	101

[a] Days from first service after parturition to conception.
[b] Days from parturition to conception.
[c] Due to failure to respond favorably to Sulmet treatment, Aureomycin was used after four or more days.

During the course of the experiment, six of the original 24 B-I cows did not respond favorably to Sulmet treatment. Eventually, these cows had to be taken off the Sulmet treatment and were treated with Aureomycin. This development had important implications in the comparison of nonantibiotic versus antibiotic

TABLE 2

Case study summary of abnormally parturient cows assigned to no-treatment group (B-III)

Cow No.	Freshening age	Condition	Retained	Treatment	Breeding efficiency S/C[a]	Breeding efficiency SP[b]	Consequence
(case)	*(yr.-mo.)*		*(days)*		*(No.)*	*(days)*	
H911	2-5	Dystocia		None			Sold—not bred
H913	2-1	Dystocia		None	1	50	Sold—pregnant
H923	2-2	Dystocia		None			Sold—paralyzed
H890	2-2	Dystocia		None	2	119	Normal calving[d]
H831	4-8	Retained	2	None	1	83	Aborted
H811[c]	5-4	Retained	3	After 4 wk.	3	102	Normal calving[d]
H807	4-4	Retained	6	None	2	111	Aborted—sterile—sold
H760	5-6	Retained	.....	None	1	80	Normal calving[d]
H689	8-6	Retained	2 or more	None			Sold—udder complications
H687	8-2	Retained	.....	After 3 days	1	82	Normal calving[d]
H671	9-7	Retained	9	After 3 days	1	78	Sold—pregnant
H669	10-1	Retained	.....	None	2	73	Pregnant
H578	11-11	Retained	6	After 6 days			Infertile—sold

[a] Services per conception.
[b] Service period, the days from parturition to conception.
[c] Treated for pyometra with antibiotics 4 and 6 wk. after calving.
[d] Normal calving at next parturition.

treatments, since none of the B-II cows required any treatment other than Aureomycin to bring about recovery. Rather than delete the six records that did not follow the treatment plan of the experiment, the data were analyzed both with the records deleted and with them included.

There were several indications that cows did not respond well to sulfonamides. Several years ago, Fincher (6) observed that sulfanilamide itself, and some other sulfa products, seemed to be irritating to the bovine uterus when affected with retained placenta or metritis.

Services per conception. Normal cows receiving Aureomycin (A-II) averaged 0.31 service higher than the A-I cows (P *ca.* 0.07), indicating that if any effect might be present it would be deleterious. The advantage of Aureomycin over Sulmet (Group B) was slightly greater (0.35 service less), but also was not statistically significant.

In an earlier study of this herd, Pfau (13) reported that cows which gave normal birth required an average of 2.10 services for the next conception, whereas cows whose calvings were accompanied by retained placenta required an average of 2.92 services for the next pregnancy. The observed improvement in breeding efficiency within this herd was undoubtedly due to a combination of factors, but one factor of importance was probably the effectiveness of Aureomycin as a method of treating abnormally parturient cows.

Nonreturn to first service. The A-II cows had a considerably lower conception rate (53%) than the A-I cows (67%). A Chi-square test of differences of nonreturns to first service showed the probability to be about 0.07, a value which was the same as found by using the method of analysis of variance on services per conception. The difference between the 46% nonreturn rate for the B-I cows and the 61% for the B-II cows was again too small for significance with the numbers available.

Service period. The A-I cows required on the average 97 days for conception as compared with 102 days for the A-II cows, a minor difference. When the mixed-treatment animals were included in the analysis, the B-II cows conceived about one estrous period sooner (24 days) than the B-I cows ($P < 0.10$).

Easterbrooks and Plastridge (5) studied the effects of Aureomycin therapy for retained placenta, using calving interval as one of the measures of breeding efficiency. By subtracting from their calving interval an average gestation length of 281 days, the resultant interval could be called the service period. On this basis, the service periods were found to be 111, 96, and 118 days for the retained-placenta cows treated every 24 hr. with 500 mg. Aureomycin, retained-placenta cows treated every 48 hr. with 500 mg. Aureomycin, and the normal cows receiving no treatment, respectively. The service periods for those cows treated with Aureomycin compared very favorably with the 96-day service period found for the cows treated with Aureomycin in this experiment.

Conception interval. Differences between treatments seemed to be most easily detected by the use of conception interval (days from first service to conception) as the measure of breeding efficiency. When the mixed-treatment animals were deleted, a difference of 11 days ($P < 0.10$) existed between A-I cows (19 days)

and the A-II cows (30 days). When the mixed-treatment animals in Group B were included, average treatment effects differed significantly ($P < 0.05$). Specific treatment comparisons revealed that the 11-day difference between A-I and A-II cows and, particularly, the 23-day difference between B-I and B-II cows, proved to be intervals of real importance (P *ca.* 0.06).

The average number of days from first service to conception for all cows in each lot ranged from 19 to 42 days, with an average of 25 days for all 232 reproductive cycles. This was considerably less than the 47-day range found by Tabler *et al.* (15) in a study of cow families. The over-all conception interval in this investigation was expected to be smaller, because the average breeding efficiency of the cows was at a higher level than in either of the two other populations studied.

Aureomycin treatment seemed to be superior to Sulmet treatment, although the observed differences in breeding efficiency were not supported by conclusive evidence because all the tested measures were not statistically significant. But in any event, all their differences were in favor of Aureomycin. Bunkhart (3) has shown that the activity of Aureomycin has a much greater spectral range than sulfonamides and that only a minimum blood concentration was necessary for therapeutic effect as compared with the greater concentration necessary for sulfonamides. If Aureomycin was superior in controlling harmful bacteria that flourish in the uterus during the condition of retained fetal membranes, or that are encouraged by injury inflicted during parturition, and at the same time caused no irritation to the uterine tissue, there is good reason to believe that cows so treated would not suffer from impaired fertility. In view of this evidence for consistent improvement of breeding efficiency, and recognizing that six cows on Sulmet treatment did not respond favorably, it seemed justifiable to attribute the better breeding efficiency to the therapeutic effects of Aureomycin.

Case studies of abnormally parturient cows (B-III). The results of this part of the experiment appear to have both practical and theoretical importance. A summary of the case studies (Table 2) showed that, from a practical standpoint, under the conditions of this experiment, all cows experiencing dystocia or retaining their fetal membranes, could simply not be neglected and exposed to the harsh laws of nature. Some cows manifested their unhealthy state within a few days after freshening and required immediate treatment, to survive. Other abnormal cows receiving no treatment at parturition did not appear to be adversely affected immediately but were possibly affected later through impaired fertility. Actually, only six cows followed the no-treatment plan of the experiment and conceived later on, providing some criteria for measuring breeding efficiency. In addition to the small number of observations, there was the problem of distinguishing clearly between normally and abnormally parturient cows.

There can be no question that some cows must be treated, to check the septic condition which may develop. In severe cases, death would have resulted if those animals had not been placed under veterinary care. Of the nine retained placenta cases, at least three cows fell into this category. Four of the nine cows eventually required treatment. This figure was considerably higher than Palmer (11)

found in his clinical no-treatment study of 44 retained placenta cases, where only 9% became septic and required treatment. The wide variation in the length of time (range three to nine days) before treatment was required indicated the individuality of each cow's condition. As Chambers *et al.* (4) pointed out, seldom was the degree of infection the same for the different cows experiencing retained placenta. Therefore, it was illustrated that the best recovery could be attained only when each case was handled individually under close observation.

The importance of dystocia is often underrated as a source of impaired fertility in the next reproductive cycle. Four of the 13 abnormal parturitions (Table 2) were classified as dystocia cases and all occurred in first-calf heifers. These observations supported Henderson's (7) findings, that the greatest proportion of all dystocia cases was found in first parturitions. This was expected, when consideration was given to all those females that were eliminated because of physical malformation or other factors causing serious damage at parturition and, therefore, did not have a chance to express this difficulty at a later calving.

Breeding efficiency information from Group B, Lot III, supported few definite conclusions. It appeared that all the cows had good breeding efficiency on the basis of conception rates, although, of the nine animals that did conceive again, three animals aborted after receiving no treatment at parturition and one of these became sterile. Conversely, some cows did not appear to be adversely affected through no treatment. The inability to determine accurately to what degree cows can depart from normal before being considered abnormal undoubtedly accounted for some of the variation in response to no treatment.

It may be concluded that cows with abnormal parturitions, retained placenta, or dystocia, should be given individual attention and, in many cases, must have treatment in order to survive and/or maintain satisfactory breeding condition. Intrauterine oblets of Aureomycin appeared to be superior to Sulmet for this treatment and cows treated with Aureomycin had breeding efficiency comparable with cows having no abnormalities at parturition.

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THYROID FUNCTION OF MONO- AND DIZYGOUS CATTLE TWINS[1]

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SUMMARY

Several indices of thyroid function were evaluated at various times of the year in three sets of cattle twins, of which two were classified as monozygous and one as dizygous on a phenotypic basis. The phenotypic diagnosis of monozygous twins based on morphological characters agreed well with the diagnosis by the determination of thyroid function. Similar observations were recorded when the twins were under different hormonal therapy. It is suggested that determination of thyroid function during the winter months (cold environment) might greatly facilitate an objective diagnosis of monozygocity.

The value of monozygous dairy cattle twins in physiological and nutritional studies has been recognized since the pioneer studies in Sweden and New Zealand. The recent reviews by Hancock (2) and Brumby (1) summarized the current information on the use of twin cattle in experimental work. The identical genetic constitution of monozygotic, or one-egg, cattle twins permits one to assess more accurately the influence of several environmental and endocrine factors upon the performance of dairy cattle independent of the genetic effect; in certain instances, they are also of great value from an economical standpoint, as reduced numbers of twins are required in experimental work, due to their greater uniformity for many productive characteristics.

There were three sets of twins in the herd at the Missouri Station, of which two pairs were suspected of being monozygous and one pair dizygous on a phenotypic basis, as indicated by morphological characteristics (body conformation and head shape). Thyroid function was evaluated in the three pairs of twins at various times of the year and also under different hormone therapy. The present report deals with the performance of the twins under the conditions mentioned above.

EXPERIMENTAL PROCEDURE

All the twins in the experiment (1–1.5 yr. of age) belonged to the Jersey breed and were kept in a dry lot, except when milked. Hay and a concentrate mixture were fed throughout the year to avoid changing pasture conditions. A block of iodized salt was always available. Three hundred microcuries of carrier-free radioiodine (NaI^{131}) were injected intravenously into each animal at the beginning of the experimental period and thyroidal I^{131} measurements were made daily by the technique of Pipes *et al.* (5). The indices of thyroid activity were the uptake of I^{131}, the normal release of I^{131} from the thyroid ($k'4$), the net release of I^{131} under a goitrogen ($k''4$) and, finally, the thyroxine secretion rate. Estrogen and

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progesterone used in the experiment were dissolved in olive oil prior to subcutaneous injection.

RESULTS AND DISCUSSION

Uptake of I^{131}. A study of the I^{131} uptake during the course of the year revealed a much greater similarity in the sets of Twins G41 and G42 and 158 and 159 than in 24 and 76 (Table 1). Hence, it appears unlikely that Twins 24 and 76 can be monozygous; whereas, the reverse could be true of Twins G41 and G42 and 158 and 159. Nearly identical I^{131} uptake has been reported in monozygous twins by Swanson *et al.* (8). However, the uptake of I^{131} has been shown to be a poor criterion of thyroid function in cattle by Lodge *et al.* (3) and Swanson *et al.* (7), and wide variations in uptake of I^{131} during the course of the year apparently unrelated to temperature have been found in our work, also.

Release of I^{131} from the thyroid gland. I^{131} release measurements are generally regarded as better indices of thyroid function than the uptake of iodine, since they are more directly related to hormone secretion. The method of determining the rate of release of I^{131} from the thyroid gland has been amply described by Pipes *et al.* (5). As in the case of uptake, the suspected dizygous twins showed much more variability in the rate of release of I^{131} from the thyroid ($k'4$) than the other two sets of twins (Table 1). A similar trend was also noticed when the rate of release of I^{131} from the thyroid gland under a goitrogen (to prevent recycling of I^{131} from the metabolized hormone) was compared (Table 1). The uptake and release studies seem to agree with the phenotypic observation that Twins 24 and 76 are dizygous. Since very similar values for uptake and release of I^{131} have been reported in identical twins by Swanson *et al.* (7), our similar values for the uptake and release of I^{131} in Twins 158 and 159 and G41 and G42 strongly suggest that they might be identical.

Thyroxine secretion rates. The rates of release of I^{131} from the thyroid gland do not measure thyroid gland activity very accurately and discrepancies between release rates and thyroxine secretion rates in cattle have been noted by Premachandra *et al.* (6). The best method available today for assessing thyroid gland function is the determination of the thyroxine secretion rate by substitution or replacement technique, which consists in the daily injection of increasing amounts of purified l-thyroxine, with concurrent administration of thiouracil, the amount being increased every third day until the release of I^{131} is prevented by inhibiting the discharge of thyrotropic hormone. The thyroxine secretion rates in Twins G41 and G42 were almost similar throughout the year, and a seasonal variation in thyroxine secretion rate was readily apparent in all the twins (Figure 1). A maximum of only 8.3% difference in thyroxine secretion rate was noted in a few estimations (Table 1). In the other pair, 158 and 159, thyroxine secretion rates were similar in all the estimations. But thyroxine secretion rates were found to differ markedly in Twins 24 and 76, and differences ranging from 20–60% were noted in the several estimations made at various times of the year (Table 1). This difference is striking when compared with a maximum of only 8.3% difference noted in the other twins. From these data, it is concluded that Twins G41 and G42

TABLE 1

Comparison of seasonal thyroid function tests in three sets of cattle twins

Month	Twin G41				Twin G42			
	U	k′ 4	k″ 4	Tx	U	k′ 4	k″ 4	Tx
Jan.-Feb.	33.6 ± 1.14	.0019 ± .0001	.0039 ± .001	0.6	35.9 ± 1.55	.0015 ± .0004	.0038 ± .002	0.55
Mar.-Apr.	30.2 ± 1.54	.0009 ± .00009		0.6	34.6 ± 1.7	.0022 ± .0001		0.55
May-June	62 ± 3.8	.0023 ± .0008	.0042 ± .0011	0.15	59.5 ± 2.7	.0033 ± .0007	.0032 ± .0009	0.15
July-Aug.	32.1 ± 1.8	.0008 ± .0001	.0024 ± .0008	0.15	31.5 ± 2.1	.0016 ± .0009	.0027 ± .0009	0.15
Sept.-Oct.	42.6 ± 2.3	.0019 ± .0001	.0048 ± .0002	0.2	41.2 ± 2.0	.0038 ± .0002	.0065 ± .0003	0.2
Nov.-Dec.	54 ± 2.6	.0024 ± .0009	.0039 ± .0006	0.2	62 ± 3.2	.0024 ± .0008	.0038 ± .0005	0.2
		Twin 158				Twin 159		
Jan.-Feb.	35.0 ± 1.29	.0044 ± .0009	.0040 ± .0010	0.6	30.8 ± 1.32	.0033 ± .0008	.0030 ± .0011	0.6
Mar.-Apr.	38.0 ± 1.3	.043 ± .001		0.6	30.6 ± 1.5	.0032 ± .001		0.6
May-June	62.8 ± 2.3	.0066 ± .0008		0.25	64.6 ± 3.3	.006 ± .001		0.25
July-Aug.	46.0 ± 1.4	.0008 ± .0001	.0057 ± .0008	0.2	48.0 ± 1.6	.0017 ± .0009	.0055 ± .001	0.2
Sept.-Oct.								
Nov.-Dec.	30.6 ± 2.0	.0032 ± .0009	.0048 ± .001	0.5	38.6 ± 1.8	.004 ± .0004	.0046 ± .0009	0.5
		Twin 24				Twin 76		
Jan.-Feb.	31.2 ± 1.49	.0025 ± .0007	.0089 ± .0006	0.7	22.1 ± 1.60	.0012 ± .0009	.005 ± .0008	0.5
Mar.-Apr.	24.2 ± 1.9	.0068 ± .0012		0.6	50.7 ± 1.9	.0006 ± .001		0.5
May-June	49.0 ± 1.9	.0071 ± .0012	.0084 ± .0004	0.20	86.0 ± 1.5	.0038 ± .0009	.0046 ± .0007	0.15
July-Aug.	32.7 ± 1.9	.0028 ± .0001	.0068 ± .001	0.15	32.0 ± 1.8	.001 ± .0001	.0031 ± .0009	0.15
Sept.-Oct.	39.7 ± 2.8	.0073 ± .001	.0155 ± .001	0.5	47.0 ± 3.2	.002 ± .0009	.0058 ± .002	0.2
Nov.-Dec.	29.4 ± 1.1	.0028 ± .0009	.0087 ± .0008	0.5	27.3 ± 2.2	.0012 ± .0009	.005 ± .0006	0.4

U—I^{131} uptake (% of administered dose); k′ 4—normal release of I^{131} from the thyroid gland (%/hr); k″ 4—net release of I^{131} from the thyroid gland when the recycling of I^{131} from the metabolized hormone has been blocked by a goitrogen; Tx—thyroxine secretion rate (mg/100 lb. body weight).

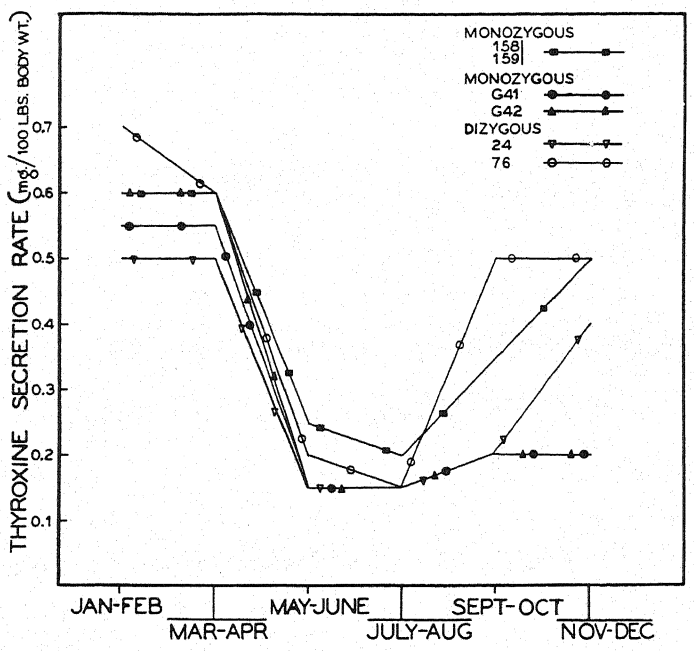


FIG. 1. The seasonal trend in thyroxine secretion rate of cattle twins at the Missouri Station is shown. The pronounced disparity in thyroxine secretion rates of dizygous twins in the winter season can be clearly seen.

and 158 and 159 are monozygotic and Twins 24 and 76 dizygotic. However, it appears that Twins 24 and 76 show greater degrees of similarity in these thyroid function tests than do unrelated animals.

Effect of estrogen and progesterone, and progesterone. Some of the heifers in the herd received estrogen and progesterone in a ratio of 1 : 1,000 in connection with an experiment to stimulate mammary gland growth. It was considered interesting to study how these twins would react to different hormonal treatments. For this purpose, 300 μc. of NaI^{131} were injected intravenously into each animal and thyroidal measurements were made according to the method already described. After obtaining a control period for normal release for five days, 100 μg. of estrogen and 100 mg. of progesterone were injected subcutaneously daily in all the twins for five days and the release rates of I^{131} determined. Under this treatment, also, the release rates were nearly equal in Twins G41 and G42 and 158 and 159 (Table 2). Greater differences were noticed in Twins 24 and 76 (Table 2). A similar pattern of behavior was noticed when 100 mg. progesterone alone was administered daily for six days (Table 2). Estrogen and progesterone, and progesterone administered at the above levels, had no effect on the normal thyroid hormone release rate. Incidentally, this level of progesterone (i.e., 100 mg/day) was assumed as a physiological one, since McDonald *et al.* (4) had shown in the bovine that pregnancy could be maintained at this level in the absence of corpora lutea.

From the above discussion, it is readily apparent that the phenotypic diag-

TABLE 2
Normal release of I^{131} (k' 4) in cattle twins during hormonal therapy

Twin No.	Estrogen and progesterone[a]		Progesterone[b]	
	Before	After	Before	After
G41	.0029 ± .0001	.0028 ± .0004	.0040 ± .0010	.0043 ± .0008
G42	.0022 ± .0002	.0026 ± .0008	.0043 ± .0010	.0046 ± .0010
158	.0029 ± .0001	.0032 ± .0003		
159	.0033 ± .0009	.0032 ± .0008		
24	.0068 ± .0008	.0070 ± .0010	.0070 ± .0010	.0069 ± .0009
76	.0006 ± .0001	.0015 ± .0009	.0048 ± .0006	.0050 ± .0009

[a] 100 μg. estrogen and 100 mg. progesterone/day/animal.
[b] 100 mg. progesterone/day/animal.

nosis of the twins has agreed well with the diagnosis by several indices of thyroid function and, in particular, the release rates of I^{131} from the thyroid gland and the actual thyroxine secretion rate. However, determination of thyroxine secretion rate in winter holds out better promise in the diagnosis, since an opportunity will be provided for the expression of the full genetic potential, and any relative inherent deficiencies in thyroid function to combat cold will be exposed. This is evidenced by the fact that thyroxine secretion rates in suspected dizygous twins are the same at the height of summer (July-August, Table 1), which implies that differences in thyroid function are not well brought out when a lowered plane of thyroid activity is the order. Since the number of sets employed in the investigation was very limited, it can not be asserted categorically that determination of thyroid function can be used as the absolute standard in the diagnosis of monozygocity. If thyroid functional tests are determined with phenotypic diagnosis (morphological characters), the relative merits of the former index in the diagnosis of monozygocity in cattle can be evaluated. From the close agreement between phenotypic diagnosis and the diagnosis by determination of thyroid function, it is tempting to suggest that thyroxine secretion rate determination in winter could play a role in the objective diagnosis of monozygocity.

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EFFECT ON REPRODUCTION OF FEEDING DIETHYLSTILBESTROL TO DAIRY CATTLE[1]

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SUMMARY

Twenty-one cows from the Kansas State College purebred dairy herd and five pairs of identical-twin cows were used to determine the physiological effects of low-level diethylstilbestrol (DES) feeding on reproductive behavior. The cows were fed DES as part of their daily grain ration at levels of 0–20 mg. per 1,000 lb. of body weight.

Results indicate that the feeding of DES at levels ranging from 5 to 15 mg. had no adverse effect on estrous cycle, conception, or gestation. Three cows receiving the 20-mg. level of DES feeding aborted; however, several other cows fed at this level had no reproductive difficulty.

There were no observable detrimental side effects associated with DES feeding.

In recent years, supplementing feeds with diethylstilbestrol (DES) has become economically important to the beef industry (3, 11). Due to possible harmful effects on the reproductive system, DES-containing feeds have been recommended only for fattening cattle. In many areas, cattle owners maintain large numbers of commercial cattle for fattening and small breeding herds of either beef or dairy cattle. Many of these livestock producers feed DES-containing feeds to their fattening cattle and would like to know the effect this additive might have on breeding stock.

This study was undertaken to determine the effects on reproductive function of feeding low levels of DES to dairy cattle.

Data on virgin goats brought to lactation by injections of 1.5 mg. of DES per week failed to show any harmful effects upon their subsequent breeding performance (4). Lewis and Turner (8) reported that there was no effect on the normal function of the ovaries in goats receiving 0.25 mg. of DES per day for long periods. They also found that treatment during the last half of pregnancy seemed to cause no harmful effects. Several workers (5, 6, 9) found that the ovaries of cows became inactive shortly after initiation of treatment with estrogens, either by injection of 170–530 mg. or by implantation of 0.5–5 g. Most of the animals exhibited irregular sexual activity without accompanying

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ovarian function. Also, there were symptoms of nymphomania, such as raised tail head, relaxed pelvic ligaments, swollen vulva, and vaginal discharge, exhibited by all the treated animals to varying degrees. Folley and Malpress (5) and Folley *et al.* (6) found on removal of the implanted estrogen tablets that multiple large follicles or cysts occurred in one or both ovaries. This, in the majority of cases, was followed by a return to normal cyclic functioning of the ovaries. Parks and Bellerly (10) found that injections of estrin during early pregnancy invariably caused rapid reappearance of estrus and termination of pregnancy in laboratory animals. The amount of estrin required for these results varied, but was approximately twice as much as required if the animals were in late pregnancy. Relatively large dosages of orally administered estrogenic compounds seem to be required to cause abortion in cattle. Folley *et al.* (7) gave a subcutaneous injection of 1 g. of stilbestrol dipropionate in 22 ml. of water to each of three pregnant Ayrshires, causing two to abort within 5 wk. and the other to give birth to an 8-mo. fetus. These workers also reported a low rate of conception in some cows following various estrogen treatments. Reuber (12) reported that feeding 10 or 12 mg. of stilbestrol per day in a fattening ration had no effect on estrous cycles or on conception.

EXPERIMENTAL PROCEDURE

Thirty-one cows including five pairs of identical twins, some not pregnant and others in various stages of gestation and lactation, were used. The cows were fed either 0, 5, 10, 15, or 20 mg. of DES/1,000 lb. of body weight daily.

Some of the cows used were slaughtered after the start of the experiment, to provide anatomical, histological, and reproductive data. The others were allowed to calve and were used to study the effects of DES feeding during following gestations.

The cows received regular herd treatment except that they were turned into a separate dry lot and received no pasture. An examination of the reproductive organs by rectal palpation was made on each cow at the start of the experiment. The cows were artificially bred as soon as they came to heat after the start of the experiment, provided they had calved at least 60 days previously. The management of the identical twins was essentially the same and has been reported previously (2).

Reproductive organs of the cows were examined per rectum at weekly intervals, for approximately 60 days following the initiation of DES feeding, and at monthly intervals thereafter.

Cows were observed daily for indication of estrus. Any abnormal behavior such as nervousness, bellowing, attempts at mounting other cows, or willingness to stand to be mounted was noted. The vulva was examined daily for appearance of mucous discharge, hyperemia, and swelling of the *labia majora*.

The reproductive organs and adrenal and pituitary glands were removed from all slaughtered animals. Gross and histological studies of the tracts and glands were made to determine any abnormalities.

All cows were fed average quality alfalfa hay ad libitum and sorgo silage at approximately 2 lb. per 100 lb. of body weight daily. A 17% protein concentrate mixture was fed at 1 lb. for each 3.5 lb. of 4% FCM produced.

DES was provided in a commercial premix.[3] The treated cows received DES once daily as part of the concentrate mixture.

RESULTS

Herd cows. A summary of the data collected on 36 gestations of 21 cows is shown in Table 1. Of the 21 cows receiving DES during the first experimental gestation, 13 were pregnant at the beginning of DES feeding. Four of these cows (126B, 112B, 197A, 180A) were fed DES continuously from the beginning of the experiment until parturition, and four cows (123B, 277A, 127B, 128B) were fed stilbestrol continuously from the start until the termination of the lactations, but were not fed stilbestrol during the dry period. Each of these eight pregnancies terminated with the birth, without difficulty, of a normal healthy calf. Four of the 21 cows pregnant at the initiation of treatment were sacrificed prior to calving. Apparently normal embryos were recovered from each cow. One cow (171A), receiving DES at the 20-mg. level, aborted a 150-day fetus after receiving DES 79 days.

Eight herd cows were fed DES prior to conception during the first experimental gestation. One cow (125B) was bred twice after the beginning of DES feeding. This animal conceived 64 days after the initiation of DES feeding and calved normally after receiving DES 288 days. Another, (268A), received DES 90 days before conceiving to the second service and calving normally after receiving DES 290 days. Three other cows (482A, 354B, and 130B) were bred and conceived after the beginning of the experiment. Apparently normal embryos were recovered from these cows at slaughter. The average number of services per conception in the College herd during this period was 2.3.

Two cows (239B, 419B) were bred one and two times, respectively, following the initiation of DES feeding. No embryos were recovered from these cows at slaughter. Cow 247B conceived to the first service after having received DES for 35 days. This cow aborted at 190 days after receiving 20 mg. of DES per 1,000 lb. of body weight daily 225 days.

The effects on conception of feeding DES in subsequent lactations are shown in Table 1. Of the nine treated cows (123B, 125B, 126B, 127B, 128B, 112B, 277B, 197A, 180A) which were bred in their second experimental lactations, five (123B, 125B, 126B, 180A, 197A) conceived after the first service. Of the other four, 127B and 128B conceived after two services, 112B after three, and 277A did not conceive after nine services.

As shown in Table 1, 123B conceived after the first service, which was nine days after the initiation of treatment. This cow was on the 20-mg. level and received 26 mg. of DES in its feed per day. This animal aborted on the 140th day of gestation. Tests for brucellosis showed that this disease was not likely re-

[3] Supplied by Eli Lilly and Company, Indianapolis, Indiana.

TABLE 1

Summary of reproductive data for herd cows fed diethylstilbestrol (DES)

Cow[a] No.	Level of DES fed daily	Days pregnant at start of experiment	Days fed DES prior to conception	Total days fed DES	No. of services to conception: Pre-treatment	No. of services to conception: During treatment	Remarks[b]
	(mg/1,000 lb. body wt.)						
123B	5	150	0	75	7	0	C.N.
	20	0	10	150	0	1	Aborted 140 days
277A	10	96	0	127	4	0	C.N.
	10 (105 days) and 20 (198 days)	0		303	0	9	N.P.S.
482A	10	0	72	114	2	2	P.S. 42 days
197A	15	148	0	129[d]	1	0	C.N.
	10	9	0	105	1	0	C.N.
	20	0	120	160	0	2	C.N.
	20	0	53	82	0	2	P.S. 180 days
354B	15	0	82	114	0	3	P.S. 32 days
419B	15	0		114	0	2	N.P.S.
268A	10 (105 days) and 15 (185 days)	0	90	290	0	2	C.N.
147B	15	30	0	53	4	0	P.S. 83 days
396B	15	63	0	53	3	0	P.S. 116 days
45[c]	15	7	0	112	1	0	P.S. 119 days
127B	20	100	0	120	1	0	C.N.
	20	0	59	261	0	2	P.S. 202 days
180A	20	9	0	294[d]	1	0	C.N.
	20	0	37	263	0	1	C.N.
	20	0	25	68	0	2	P.S. 43 days
163A	20	70	0	169	1	0	P.S. 239 days
171A	20	71	0	79	2	0	Aborted 150 days
128B	20	14	0	211	1	0	C.N.
	0			0	2	0	C.N.
130B	15	0	22	53	2	1	P.S. 31 days
247B	20	0	35	225	0	1	Aborted 190 days
239B	15	0		45	0	1	N.P.S.
125B	5	0	64	288	0	2	C.N.
	15 (208 days) and 20 (84 days)	0	14	292[d]	0	1	C.N.
126B	5 0 15 (114 days) and 20 (60 days)	0	54	174	0	1	C.N.
	20	0	30	65	0	1	C.N.
112B	10	97	0	185[d]	1	0	C.N.
	15	0	110	330	0	3	C.N.
	20	0	117	152	0	4	C.N.

[a] No. 100-199 = Holstein; 200-299 = Ayrshire; 300-399 = Jersey; 400-499 = Guernsey.

[b] C.N.—Calved apparently normally, N.P.S.—not pregnant at slaughter, P.S.—pregnant at slaughter.

[c] Milking Shorthorn.

[d] Fed DES until parturition.

sponsible for the abortion. However, there were several abortions in the herd during this time and some of these were definitely attributed to leptospirosis. Four of the others (197A, 180A, 125B, 112B) that received DES throughout the lactation calved normally. Two (126B, 128B) that did not receive DES during the complete lactation also calved normally. An apparently normal 202-day fetus was recovered at the slaughter of 127B.

Four of the herd cows received DES in their third successive lactations. Three (126B, 180A, 197A) reequired two services or fewer for conception, and the other (112B), four services. A normal 43-day fetus was recovered at the slaughter of 180A and the three other cows calved normally. Two of these cows (126B, 197A) were fed DES in their fourth successive lactations and conceived after one and two services, respectively. Cow 126B calved normally and an apparently normal 180-day fetus was recovered at the slaughter of 197A. DES feeding was discontinued shortly after conception in all these cases.

Identical twins. The results obtained with the identical twin cows are shown in Table 2. Treated cows, 82, 83, 195, and 41, conceived after first service; therefore, the average number of services per conception in the treated group of twins was 1.0. The average number of services per conception in the control group was 2.0. These figures do not include the five breedings of experimental cow 85 or control cow 84. Breeding failures in Cow 84 were found to have been

TABLE 2

Summary of the effect diethylstilbestrol (DES) had on the reproductive performance of identical twin cows during two consecutive gestations

Cow No.	Level of DES fed daily	Times bred	Av. length[a] of estrus cycle	No. days fed DES prior to conception	Total days DES fed	Remarks[b]
	(mg/1,000 lb. body wt.)		*(days)*			
				Lactation I		
81	0	2	23.1	0	0	C.N. in 281 days 61 lb.
82	10	1	22.6	59	276	C.N. in 282 days 63 lb.
84	0	5	19.7	0	0	N.P.S. oviducts occluded
83	10	1	19.0	30	112	Pregnant 92 days at death
86	0	1	25.0	0	0	C.N.
85	10	5	21.8	—[c]	179	N.P.S.
194	0	2	23.5	0	0	C.N. in 280 days 82 lb.
195	10	1	27.0	75	288	55 lb. in 270 days
40	0	2	24.6	0	0	C.N. in 284 days 97 lb.
41	10	1	21.5	10	240	C.N. in 281 days 85 lb.
				Lactation II		
81	0	1	23.6	0	0	C.N. in 280 days 58 lb.
82	10	1	24.5	58	147	C.N. in 281 days 50 lb.
194	0	6	24.5	0	0	Abort. 117 days
195	10	6	21.7	175	245	P.S. 138 days
40	0	2	26.0	0	0	C.N. in 287 days 78 lb.
41	10	1	21.0	39	249	C.N. in 276 days 86 lb.

[a] This includes cycles prior to breeding.
[b] C.N.—Calved apparently normally, N.P.S.—not pregnant at slaughter, P.S.—pregnant at slaughter.
[c] Did not conceive.

caused by oviductal obstructions. The reason Cow 85 failed to conceive is not known; however, it was abnormal after calving, showing estrous activity every four to six days for approximately 60 days.

Treated cows 82, 83, 195, and 41 conceived 59, 30, 75, and ten days, respectively, after the inception of DES feeding. An accident caused the death of 83 after 4 mo. of treatment. An apparently normal embryo was recovered at its death. The other three pregnancies resulted in the birth of normal calves.

Three twins (82, 195, 41) were fed DES in the next lactation. Cows 82 and 41 conceived after the first services, whereas their pair mates conceived after one and two services, respectively. A normal calf resulted from each of these pregnancies. Both twins (194, 195) of the third pair required six services for conception. The control twin (194) aborted at 117 days and the treated cow had an apparently normal 138-day fetus at slaughter.

Effect on estrus. Approximately 20 to 30 days following the initiation of DES feeding, there was a tendency for the vulvas of both pregnant and nonpregnant cows to become swollen and hyperemic. This condition could have been mistaken as an indication of estrus; however, it persisted only about 2 wk. and during this time it did not interfere with normal ovarian function. There was no evidence of nymphomania at any time during the experiment in cows being fed DES. The ovaries, as determined by rectal palpation, remained functional throughout the experiment. Also, the ovaries of all the DES-fed cows slaughtered during the course of the experiment showed no indications of abnormal function. While length of estrous cycles varied among treated cows, the variation was no greater than that normally observed in dairy cattle or in control animals.

There was no evidence of elevated tailheads, relaxed pelvic ligaments, or sunken loins in any of the cows receiving stilbestrol.

The pituitary, adrenal, and thyroid glands of DES-fed cows were weighed and measured immediately after slaughter. No evidence of glandular abnormality was noted when these data were compared with weights and measurements of glands obtained from control animals.

DISCUSSION

The results of this experiment show rather conclusively that the reproductive behavior of dairy cows fed DES at levels varying from 5 to 15 mg. per 1,000 lb. of body weight per day will not be affected in the same manner as reported for beef or dairy cattle receiving injections or implants of DES.

Numerous reports of undesirable side effects accompanying the administration of DES to livestock are found in the literature (1, 11, 13). The most important detrimental manifestation concerning reproduction, usually accompanying DES administration, is the inactivation of the ovaries. However, inactivation of ovaries was not found to take place in beef heifers fed DES at levels of 5 to 10 mg. per day (3). So far as could be determined, there was no effect whatsoever on the ovaries of any of the dairy cows fed DES during the course of this study. This lack of influence on ovarian function may be due to the small amount of estrogenic material that is absorbed and made available to

exert a physiological response. Other points affirming the lack of effect on the ovaries were the normal cycling and conception of the treated animals.

A major concern to the practical aspect of DES administration to cattle is its effect on pregnancy. The number of cases studied in this experiment indicate that 5–15 mg. per day of DES orally administered to dairy cattle has no harmful effect on pregnancy. Possibly, it is not safe to feed 20 mg/1,000 lb. of body weight of DES daily to pregnant cows, since three on the 20-mg. level aborted during the period of treatment. However, it is not possible to attribute these abortions definitely to DES feeding, since abortions occurred in untreated animals of the College herd during the time of the experiments and leptospirosis was a herd problem.

The only problem routinely encountered in this experiment regarding reproductive behavior was the hyperemia of the vulva in most of the treated animals. This condition did not persist but when present it simulated symptoms of estrus. The degree of hyperemia varied with animals and apparently was not directly proportional to the level of estrogen administered.

Another factor leading to the postulation that only small amounts of stilbestrol entered the systematic circulation of the experimental animals of this study is the lack of effect on the physical appearance, and the normal appearance of the pituitary and adrenal glands.

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USE OF RADIOACTIVE CHROMIUM OXIDE IN DIGESTIBILITY DETERMINATIONS

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SUMMARY

Radioactive chromium oxide was tested as a digestibility indicator in dairy cattle by making direct comparisons of component digestibility coefficients with total collection procedure and chromium oxide ratio technique. The use of a radioactive isotope as a digestibility reference substance was found to save time and labor and, under the conditions of this experiment, to display about the same degree of precision as the two other methods studied. A disadvantage to the use of isotopes in this type of study is the increased care and expense involved in the disposal and handling of animals and excreta, as dictated by current Atomic Energy Commission and Food and Drug regulations.

Various ratio techniques have been used to measure the digestibility of forages and feeds in this and other laboratories. The development of the use of Cr_2O_3 in the ratio technique, for determining digestibility in place of the conventional total collection procedure, has proved to be advantageous. To the authors' knowledge, no radioactive substance has been utilized to determine the digestibility of nutrients fed to ruminants. Radioactive isotopes applied to digestibility studies suggest savings in time and labor, by substituting the automatic reading of samples for the relatively slow chemical Cr_2O_3 assay, and increased precision, by reducing the amount of error associated with the chemical analysis of Cr_2O_3.

The present investigation was undertaken to determine the efficiency and feasibility of using radioactive chromium oxide as an indicator in digestibility studies. The plan of the experiment was a series of four digestion trials, in three of which direct comparisons of digestibilities obtained by different methods would be made. The first digestion trial, in which neither ordinary nor radioactive chromium oxide was fed, served as a control. The three digestibility methods used were: (a) the standard total collection procedure; (b) chromium oxide ratio technique; and (c) ratio technique in which the radioactivity of $Cr_2{}^{51}O_3$ was used to calculate digestibility.

EXPERIMENTAL PROCEDURE

Two Holsteins and a Jersey were fed rations of alfalfa hay and alfalfa hay plus starch. The alfalfa hay fed was graded as U.S. No. 1 according to U.S. standards of hay grades. The starch fed was a commercial cornstarch, Amazo. Daily supplements of 100 g. steamed bone meal and 50 g. salt also were provided. In Table 1, the amounts of hay and starch fed in the different periods are given.

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[1] Data presented are part of a thesis presented by the senior author in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the Graduate School of Georgetown University, Washington, D. C., 1957.

TABLE 1
Daily rations fed (in pounds)

Period	I			II			III			IV		
Cows	280	293	647	280	293	647	280	293	647	280	293	647
Alfalfa hay	32	32	25	30	30	23	28	28	21	28	28	21
Starch	0	0	0	6	6	6	0	0	0	1–6	1–6	1–6

The animals were stanchioned in digestion stalls equipped with a sloped endless belt mechanism which automatically separated feces from urine. Collection periods were five or six days. Preliminary periods varied in accordance with objectives of another experiment; ten days in Periods I and II, five days in Period III, and zero days in Period IV. Animals were fed twice daily at 5 A.M. and 2 P.M., at which times both types of chromium oxides were administered by balling gun. The radioactive chromium oxide was given in Size No. 000 gelatin capsules, containing 300 mg. of a mixture of $Cr_2{}^{51}O_3$, Cr_2O_3, and wheat flour in the proportions 1 : 9 : 70, respectively, for Period II, and 2 : 18 : 60 for Periods III and IV. The unirradiated Cr_2O_3 was given in Size No. 10 capsules, which contained a 15-g. mixture of Cr_2O_3 (53.82%) and wheat flour.

Fecal samples from the total collection were taken daily at 9 A.M. Subsamples of the total collection feces were used for both the $Cr_2{}^{51}O_3$ and the Cr_2O_3 determinations. This procedure was adopted to reduce sampling errors to a minimum. Feces samples were dried at 80° C. to constant weight and then ground in a Wiley mill through 1-mm. mesh screen.

Official methods of A.O.A.C. (1) were used for the chemical analysis of feeds and feces. Total collection and chromium oxide ratio technique digestibilities were determined by methods previously described (5). The digestibilities reported are for alfalfa hay. An average of Morrison's (7) and Schneider's (8) digestibility values for starch was used to correct for indigestible starch residues. Digestibility coefficients were calculated by allowing a two-day lag between feed and feces for the passage of food through the animals (6).

In Period II, the daily fecal samples were pooled, and the radioactivity and chromium oxide content of the composite samples determined. In Periods III and IV, the daily fecal samples were analyzed for chromium oxide and radioactivity. Average values of the six-day periods were used for digestibility computations.

RADIOACTIVE METHOD

Chromium ^{51} oxide, the radioactive isotope used, was selected for the following reasons: (a) It has been established that chromium oxide passes through the bovine intestinal tract without absorption (3); (b) the radiation of Cr^{51} is relatively simple, mostly electromagnetic or gamma emission (2); (c) it has a short half-life of 27.8 days (2).

Chromium oxide in the amount of 1.5 g. and in the form of a fine powder was sent to Oak Ridge National Laboratories for irradiation and partial conversion to $Cr_2{}^{51}O_3$. When returned to Beltsville, the irradiated chromium oxide had a specific activity of 33 mc. per gram. Chromium oxide (13.5 g.) was added

to the activated 1.5 g. as a carrier and thoroughly mixed in a blendor. The resulting mixture was divided into lots of 5 and 10 g. and diluted with 35 and 30 g. of wheat flour, respectively. The unequal proportion of the radioactive material in the two batches was carried out in order that the amount of radioactivity fed in Periods III and IV a month later would be approximately the same level of radioactivity as in Period II.

In applying radioactive techniques to digestibility studies, it is not necessary to know the absolute amount of radioactivity fed. The relative activity of the dry matter intake and output is all that is required for the measurement of dry matter digestibility. This proportion can be determined by simply measuring the radioactivity of the feed and feces. In this study, radioactivity was determined by counting samples of infinite thickness, that is, samples of sufficient depth, so that radiation from the lowest layer is completely absorbed by the upper layers. Then, self-absorption correction is unnecessary, and the counting rate is proportional to the concentration of radioactivity in the sample. Under the conditions of this experiment, 4 g. of material were found to be a sample of infinite thickness. This amount of feed or feces under a pressure of 12,000 lb. in a Carver press produced a flat circular mat, 3.3 cm. in diameter and 0.6 cm. in depth, a size which closely approximated the area of the sample cup in the proportional counter. Since all samples were of the same size and depth, the counts recorded in the counter were proportional to the $Cr_2{}^{51}O_3$ content of the samples. A proportional counter was used to measure radioactivity.

Alfalfa hay standards were made by adding mixtures of diluted $Cr_2{}^{51}O_3$; for example, (1 $Cr_2{}^{51}O_3$: 9 Cr_2O_3: 70 wheat flour), to dried and ground alfalfa at the rate of 4 mg. of diluted radiochromium mixture to 1 g. alfalfa hay. In determining digestibility by means of radioactivity, the following formula was used:

$$\text{D.M. digestibility} = \frac{100\,[1 - (\text{c/s/mg } Cr_2{}^{51}O_3 \text{ in A.S.} \times \text{mg.} Cr_2{}^{51}O_3 \text{ fed/g D.M. alfalfa intake})]}{\text{c/s/g/feces D.M.}}$$

A.S. = alfalfa standard
D.M. = dry matter
c/s = counts per second

RESULTS AND DISCUSSION

The chemical analyses of the alfalfa hay and starch fed are reported in Table 2. Starch data are from Morrison (7).

TABLE 2
Average chemical composition of rations (% dry matter)

Ration constituent	Period	Protein	Ether extract	Ash	Crude fiber	Nitrogen free ext.
Alfalfa hay	I	17.6	1.48	7.34	25.7	47.8
Alfalfa hay	II	17.7	1.34	7.41	26.4	47.1
Alfalfa hay	III and IV	17.4	1.46	7.24	27.2	46.8
Starch[a]	II and IV	0.6		0.20	0.2	99.1

[a] Starch data from Morrison's *Feeds and Feeding* (7).

To discover the amount of the daily variation in radio-chromium oxide excretion, the radioactivity of the daily fecal samples was measured in Periods III and IV, as well as in the preliminary period of Period III. Figure 1 delineates the daily variation of the radioactivity found in the feces of the individual cows and the daily average for the three animals. The daily excretion rate of the radioactive chromium oxide displayed irregularities similar to that shown by the daily Cr_2O_3 excretion (Figure 2).

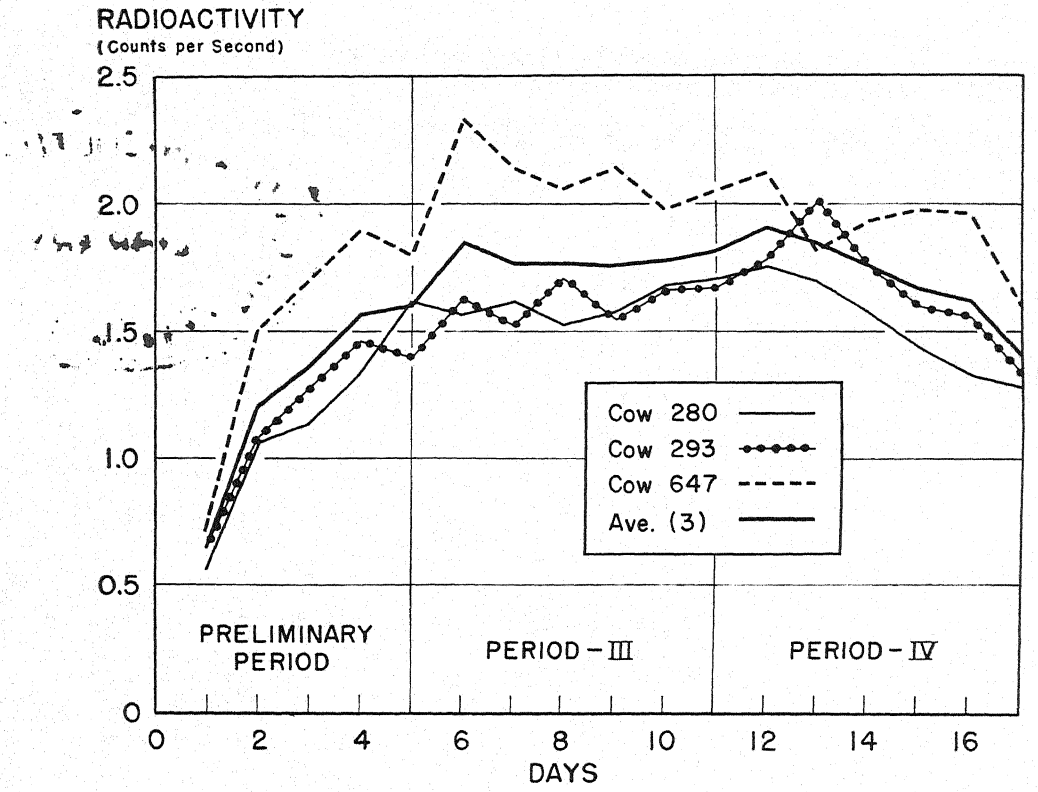
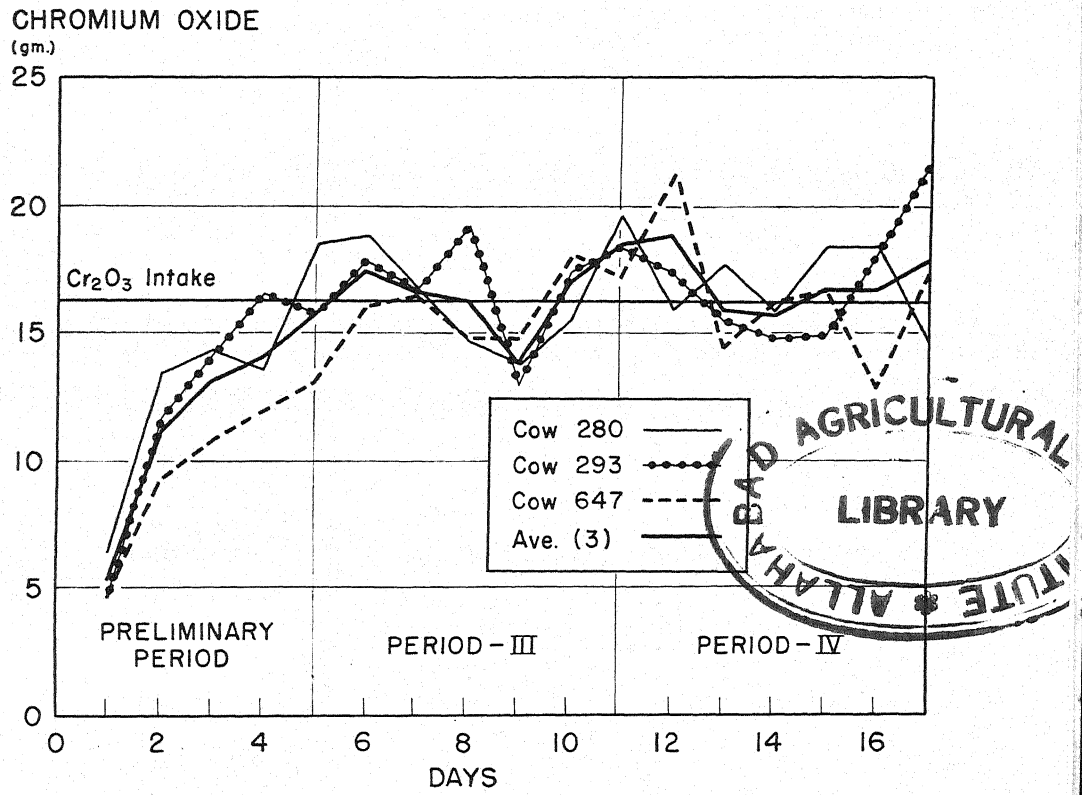


FIG. 1. Daily variation in radioactive chromium oxide excretion.

As shown in Table 3, the average apparent digestibility coefficients of the components of alfalfa hay obtained by the three methods appear to be in agreement. The magnitude of the errors involved in each procedure, indicated by standard deviations, was found to be relatively the same. This includes variations due to animals and the chromium oxide determinations. The expected increase in precision of the radioactive method did not materialize, probably due to the low efficiency of measuring radiation in samples of infinite thickness. Some information regarding the relative precision of the two methods of analyzing for chromium was obtained by calculating their coefficients of variation. Data used were the chromium oxide and radioactivity of the daily fecal samples of the three cows in Periods III and IV. The chromium oxide analyses were repli-



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FIG. 2. Daily variation in chromium oxide excretion.

cated from two to five times, while the radioactive determinations were done in quadruplicate. Data were screened for removal of determinations that could be rejected according to the criteria of Pierce and Haenisch (8). The chemical coefficient of variation was 3.0% and the radioactive was 4.4%. These results indicate that no increase in analytical precision of chromium oxide determination was attained by the use of radioactive chromium. However, results are not conclusive, since the comparative system used above favored the chemical method, which was not done in quadruplicate but repeated only enough times to get satisfactory checks. Also, the levels of radioactivity in samples were relatively low and it is likely that some increase in precision might be obtained by feeding higher levels of radioactivity.

An analysis of variance (10) of the component digestibility coefficients in Periods III and IV showed no significant differences for methods or periods, and highly significant differences for components and cows. In this statistical analysis, cows were considered random, in order to predict what would most likely happen in future replications. Had cows been fixed, periods would have been highly significant. The precision for measuring period differences in this particular statistical design was low, due to the small number of degrees of freedom (2) in the periods-by-cows interaction, the proper error term for detecting significance for periods.

TABLE 3

Component digestibility coefficients obtained by the three methods with standard deviations[a]

Period	Method	Dry matter	Cr. protein	Ash	Ether extract	Cr. fiber	NFE
I	T.C.[b]	60.2(±0.1)	69.4(±1.1)	23.1(±4.4)	14.0(±3.6)	42.3(±1.4)	73.6(±0.6)
II	T.C.	59.9(±0.8)	66.2(±3.0)	27.1(±4.6)	13.4(±8.6)	44.0(±2.0)	72.5(±0.7)
II	Cr_2O_3	59.3[c]	68.3	26.1	2.4	43.7	71.5
II	$Cr_2^{51}O_3$	61.2(±3.0)	67.4(±4.0)	29.3(±10.5)	16.9(±2.9)	46.5(±3.8)	73.5(±1.8)
III	T.C.	61.0(±0.2)	69.1(±1.3)	24.3(±3.6)	30.0(±4.9)	48.0(±1.3)	72.3(±0.2)
III	Cr_2O_3	62.0(±1.2)	69.9(±0.4)	26.3(±3.2)	31.9(±3.2)	49.1(±2.4)	73.0(±0.9)
III	$Cr_2^{51}O_3$	61.4(±0.4)	69.4(±1.1)	25.2(±4.7)	30.8(±4.7)	48.4(±1.5)	72.6(±0.31)
IV	T.C.	57.6(±1.9)	63.2(±2.3)	20.4(±4.5)	24.1(±1.8)	42.4(±4.7)	70.7(±0.8)
IV	Cr_2O_3	59.0(±1.3)	64.5(±2.2)	23.3(±4.9)	26.8(±2.4)	44.4(±4.1)	71.9(±0.6)
IV	$Cr_2^{51}O_3$	57.9(±2.4)	63.6(±3.1)	21.0(±10.2)	24.7(±8.0)	43.1(±1.3)	71.1(±1.6)

[a] Average of three cows.

[b] Total collection.

[a] Data for one cow only. Loss of intake Cr_2O_3 occurred in this period for two cows.

The following interactions were found to be highly significant: method-by-cows, periods-by-cows, components-by-cows, methods-by-periods-by-cows, and periods-by-components-by-cows. It appears that the cows reacted consistently neither to the digestibility methods used nor to the addition of starch. Nor were the components of alfalfa digested in a similar manner by the cows.

An explanation for the significance of the above interactions could be due to the differential response of the cows to the addition of starch. The accuracy of digestibility determinations, especially by ratio technique, depends to some extent on the regular flow of food residues and digestibility indicators through the intestinal tract. The addition of starch appeared to disturb the normal flow of nutrients, as evidenced by increased amounts of refused feed and wider daily variations in fecal dry matter output.

Throughout the experiment, the cows remained in apparent good health except for Cow 280. This animal bloated on March 15, Period II, as well as on April 22, 23, 24, 25, 26, 27, and 29, Period III. The bloat was a mild type, usually relieved by simple procedures such as walking the animal or standing it on a slope. Bloat apparently had no noticeable effect on dry matter digestibility or on any of the component digestibilities.

The time required for a chemical determination of chromium oxide by the Edin method averaged 1 hr. The time required for a radioactive determination of irradiated chromium oxide was about 10 min. If samples are run in triplicate or quadruplicate, it is evident that the radioactive chromium oxide method provides a saving of time, labor, and chemicals.

On the other hand, the radioactive digestibility methods have disadvantages. First, there is the necessity for providing for the segregation and disposal of excreta containing radioactive isotopes in accordance with the Federal code (4), which specifies burial in soil at a minimum depth of 4 ft. Secondly, animals intentionally fed radioactive isotopes under present Food and Drug regulations can not be sold on the open market, or returned to normal life with the herd, but must be kept segregated.

No evidence was found that the ingestion of 0.3 μ of radiation per kilogram body weight had affected experimental animals in any way.

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STRONTIUM AND CALCIUM UPTAKE AND EXCRETION IN LACTATING DAIRY COWS[1]

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SUMMARY

Sr^{89} and Ca^{45} were given orally to lactating dairy cows. From a single dose, 0.55 ± 0.05 and 1.8 ± 0.30% of the administered Sr^{89} were found in milk and urine, respectively, during a nine-day period. Under the same conditions, 3.77 ± 0.17 and 0.82 ± 0.08% of the administered Ca^{45} were found in milk and urine, respectively. The average observed ratios of Sr^{89}/Ca^{45} for four cows were: blood diet = 0.33; urine diet = 1.82; milk diet = 0.15; milk blood = 0.44, urine blood = 5.43. The excretion patterns for Sr^{89} in milk, urine, and feces can be described almost entirely by an exponential function of time.

The problem of an increasing level of Sr^{90} being incorporated into the human food chain has been quite well characterized (2, 3, 6, 8, 9). Two factors have emphasized the need for the investigation of strontium metabolism in dairy cows. First, strontium and calcium are chemically similar and react similarly in animal metabolism; secondly, milk constitutes the source of approximately 80% of the calcium in the human diet (8) and, therefore, is potentially a high source of strontium.

Although the metabolism of strontium in animals is similar to that of calcium, it has been found that in the passage from diet to milk, calcium is absorbed from the intestine and secreted into milk in larger amounts than if the two reacted chemically and metabolically the same (10). The observed ratio[2] of Sr/Ca from diet to milk for dairy cows has been reported to be 0.09 to 0.16 (4). More recently, Alexander and Nusbaum (1), using spectrographic methods, have determined the retention factor (observed ratio) for cows from diet to milk to be 0.13 ± 0.03. Using Sr^{89} and Ca^{45} double-label experiments with goats, this ratio has been reported by Wasserman *et al.* (11) to be 0.09.

Since reports of experiments in which a group of cows and in which Sr^{89} and Ca^{45} were both used seem to be lacking, this investigation was conducted to further clarify the Sr-Ca relationships in lactating dairy cows.

EXPERIMENTAL PROCEDURE

In the first of two experiments, four cows were each given orally 1.7 mc. of Sr^{89} only; in the second, four cows were each given orally 2.0 mc. of Sr^{89} and

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[2] Comar *et al.* (5). Observed ratio (sample-precursor) $= \frac{\text{Sr/Ca of sample}}{\text{Sr/Ca of precursor}}$.

2.5 mc. of Ca^{45}. All cows were maintained on an 18% protein pelleted concentrate, with grass hay as the only roughage, and were kept in elevated digestion stalls (7) for a four-day preliminary and a nine-day experimental period. Daily total weights of urine and feces were taken separately and milk weights were taken twice daily. Samples were taken for analyses at the time of weighing. Blood samples were collected daily from the jugular vein. The amount of isotope in the blood was calculated on the basis of blood being 7.7% of the animal's weight. In the first experiment, where Sr^{89} only was used, milk, blood, and urine were directly counted as liquid samples. Feces were ashed at 600° C. for 5 hr. and the ash was brought into solution and then counted as liquid samples. In the second experiment, samples were dried, ashed at 600° C. for 5 hr., brought into solution, precipitated as Sr^{89} and Ca^{45} oxalates, and differentially counted, using an aluminum absorber to distinguish between the beta energies.

RESULTS

The average amounts of Sr^{89} and Ca^{45} found in milk and urine during the nine-day period are presented in Table 1. Sr^{89} data from the two experiments were similar and are combined for presentation.

During these experiments, the average milk production for the eight cows was 8.49 ± 2.36 kg. (18.7 ± 5.2 lb.), urine excretion was 8.21 ± 2.41 kg., and feces excretion, 14.76 ± 4.79 kg. per day.

The cumulative Sr^{89} excretion patterns in milk, urine, and feces are given in Figures 1, 2, and 3. The data from eight animals were averaged to obtain the Sr^{89} excretion curves. A model was selected for each curve and the equation was derived by a least-squares analysis. The derivative of each of these equations was taken and the rate curves plotted. These equations are valid between Days 1 and 9 for feces and urine and between Days 3 and 9 for milk.

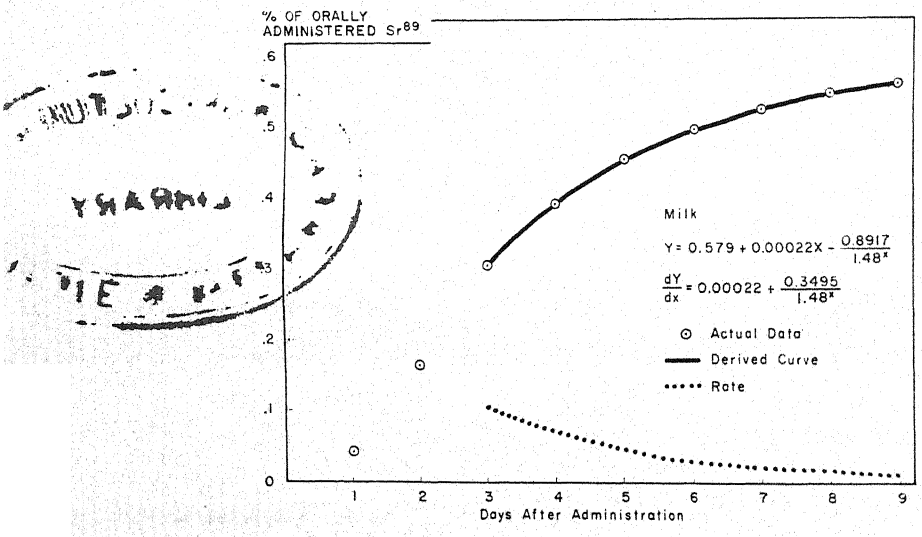


FIG. 1. Cumulative and rate of excretion of Sr^{89} in milk (average of eight cows).

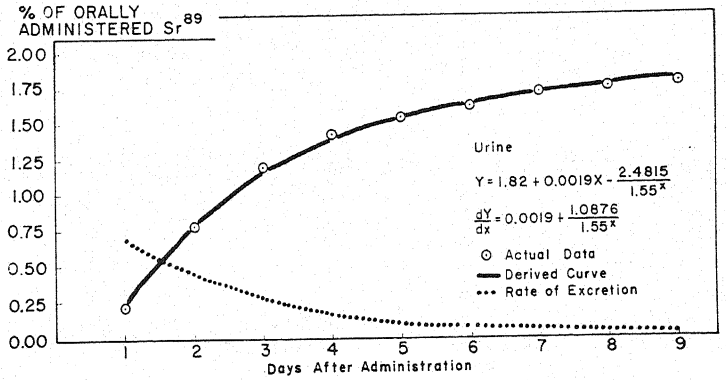


FIG. 2. Cumulative and rate of excretion of Sr^{89} in urine (average of eight cows).

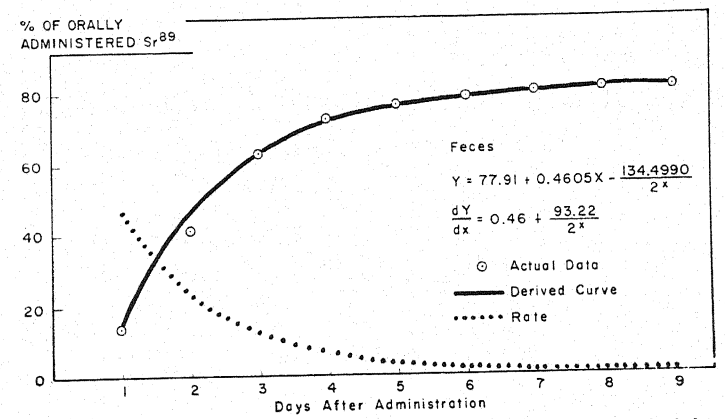


FIG. 3. Cumulative and rate of excretion of Sr^{89} in feces (average of eight cows).

The equations for Sr^{89} and their derivatives are:

$$Y\ (\text{milk}) = 0.579 + 0.00022\,X - \frac{0.8917}{1.48^x}$$

$$\frac{dy\ (\text{milk})}{dx} = 0.00022 + \frac{0.3495}{1.48^x}$$

$$Y\ (\text{urine}) = 1.82 + 0.0019\,X - \frac{2.4815}{1.55^x}$$

$$\frac{dy\ (\text{urine})}{dx} = 0.0019 + \frac{1.0876}{1.55^x}$$

$$Y\ (\text{feces}) = 77.91 + 0.4605\,X - \frac{134.4990}{2^x}$$

$$\frac{dy\ (\text{feces})}{dx} = 0.4605 + \frac{93.2193}{2^x}$$

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Where Y is the estimated cumulative excretion of Sr^{89} expressed as per cent of administered dose and X is days.

TABLE 1

Sr^{89} and Ca^{45} found in milk and urine during a nine-day period (expressed as per cent of administered dose)

	Sr^{89} (average of eight cows)	Ca^{45} (average of four cows)
Milk *(total)*	0.55 ± 0.05	3.77 ± 0.17
Milk *(per liter)*	0.066 ± 0.005	0.39 ± 0.02
Urine *(total)*	1.80 ± 0.30	0.82 ± 0.08
Urine *(per liter)*	0.26 ± 0.05	0.11 ± 0.02

Total excretion of Sr^{89} for eight cows in milk, urine, and feces averaged 83% of the administered dose for the nine-day period. Sr^{89} excretion in the feces averaged 81 ± 11% of the administered dose.

The per cent of administered dose of Sr^{89} and Ca^{45} in blood for Cows 5–8 is presented in Figure 4. Sr^{89} content averaged 0.34 of the Ca^{45} content, which is the observed ratio (blood-diet).

Observed ratios of Sr^{89}/Ca^{45} are presented in Table 2. This is a method of expressing the change in the fraction of Sr^{89}/Ca^{45} from precursor to sample. (See Footnote 2.)

On a daily basis, only the observed ratios for feces indicated a definite change. The ratios were less than one during the first four days of the experiment and became greater than one during the remainder of the experiment. This difference was significant ($P < .01$) and was attributed to a higher endogenous calcium than strontium. The observed ratio (feces-diet) for the entire period was 1.0. All

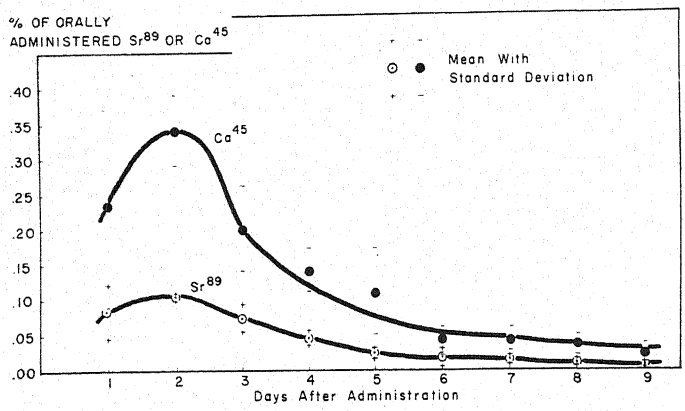


FIG. 4. Per cent of administered dose of Sr^{89} and Ca^{45} in blood.

TABLE 2

Observed ratios of Sr^{89}/Ca^{45} for four cows

Cow	Blood Diet	Urine Diet	Milk Diet	Milk Blood	Urine Blood
5	0.31	1.50	0.13	0.42	4.88
6	0.36	1.93	0.17	0.49	5.42
7	0.32	1.73	0.13	0.40	5.36
8	0.35	2.12	0.15	0.44	6.06
Average	0.34	1.82	0.15	0.44	5.43

observed ratios reported in Table 2 were determined from total Sr^{89} and Ca^{45} values over the entire period.

As supporting information, the amounts of Sr and Ca in the grain and hay were determined, using a Beckman DU flame spectrophotometer, operating at 460.7 and 626 mμ for the two respective elements. The average of four determinations showed that the grain concentrate contained 23 p.p.m. Sr and 8,695 p.p.m. Ca and that the roughage contained 13 p.p.m. Sr and 4,896 p.p.m. Ca. In terms of Sr: 1,000 Ca, values of 2.65 and 2.66 were determined for grain and hay, respectively, which are in agreement with values reported by Alexander and Nusbaum (1). With an average daily grain and hay intake of 5.45 kg. (12 lb.) and 3.72 kg. (8.2 lb.), respectively, daily intakes of Sr and Ca were 0.18 and 65 g., respectively, for each cow.

DISCUSSION

These experiments show that the observed ratio (milk diet) is 0.15. The observed ratios of blood diet (0.34) and milk blood (0.44) are the stepwise contributions to the milk diet ratio. Multiplication of the two latter factors equals the direct milk diet determination. The observed ratio (urine diet) is 1.82. Multiplication of the blood diet ratio by the urine blood ratio (5.43) equals 1.84, which compares closely with the direct determination of the urine diet ratio.

The observed ratio (milk diet) of 0.15 ± 0.02 found in this investigation, using radioactive Sr and Ca, is slightly higher than the 0.13 ± 0.03 found by Alexander and Nusbaum (1), using spectrographic methods. Although a lower ratio may occur when Sr and Ca are in their natural state in feeds, there is little evidence to support the occurrence of a different ratio when Sr^{89} and Ca^{45} are orally administered as chlorides.

The animals used in these investigations were physiologically adjusted throughout the experimental period. Although feces dry matter was not determined in these experiments, the results from subsequent experiments show it to be 23% under similar conditions. Milk production was 8.34 kg. per cow per day for the two days before placing the cows in the digestion stalls. This compares well with the 8.49 kg. average during the experiment.

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PELLETED ALFALFA HAY FOR MILK PRODUCTION

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SUMMARY

Rations containing pelleted or chopped alfalfa hay were compared when fed with and without concentrates to producing dairy cows. Cows on pelleted hay consumed more dry matter and produced more milk than those receiving chopped hay when no concentrates were fed. Supplementation of chopped hay with about 12% concentrates increased dry matter intake and production to a level comparable to that of the pelleted hay rations. Increased dry matter intake and milk production when pelleted hay was supplemented at a similar rate were not significant. The butterfat percentage of the milk was unaffected by either of the pelleted hay rations.

There is much interest for using pelleted hay in milk production rations. There is considerable concern, however, over the effect of the grinding of hay upon the butterfat percentage of the milk produced. Some limited but widely quoted work by Powell (7, 8, 9) suggested that the feeding of ground roughage depressed the butterfat percentage of the milk produced on such rations. Rations consisting of free-choice long hay and concentrates, fed according to production, were compared to rations in which ground hay and concentrates were mixed and "checkered." Although the levels of intake of long hay were not reported, the levels of ground hay appear to have ranged from 22.3 down to 1.7 lb. Concentrate intakes of the cows receiving ground hay ranged from 15.1 to 28.9 lb.; whereas, cows which were fed long hay received concentrates at much lower levels of intake, ranging from 7.6 to 13.2 lb. With the number of complicating variables involved, it is difficult from these data to incriminate the physical nature of the roughage per se as the cause of the depression of the butterfat percentage of the milk.

In the first of two trials conducted by Porter *et al.* (6), there was a slight decrease in the butterfat percentage of milk when ground and pelleted alfalfa hay was fed. Total butterfat was not affected and since milk production was increased, these results seem to be in line with the commonly accepted inverse relationship between the level of milk production and butterfat percentage. In the second trial, a real drop in butterfat percentage was observed but in this case, the consumption of roughage was much lower, apparently due to the hardness of the pellet used.

More recently, Jones *et al.* (3) reported a drop in butterfat percentage from 4.13% on baled alfalfa hay to 4.00% on pelleted alfalfa hay, with the difference being significant statistically. There may be some doubt relative to the importance of this difference, however, since there was no apparent effect either upon the amount of actual milk or upon the 4% FCM produced.

The present report presents the results of a study in which no effect upon the butterfat percentage of milk was observed when pelleted or chopped alfalfa hay was fed.

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EXPERIMENTAL PROCEDURE

Twelve cows with potential production of 400 lb. butterfat were selected and allotted to a multitreatment, switch-back design as developed by Lucas (4). Four treatments were used, such that pelleted and chopped hay could be compared with and without the feeding of concentrates.

Baled alfalfa hay containing about 53% TDN, as estimated by the technique of Meyer and Lofgreen (5), was used in this study. The hay was prepared for pelleting by grinding in a hammermill through a 3/16-in. round-hole screen. The ground material was compressed without steam or water in a California, heavy-duty pellet mill with a 3/8-in. round-hole die. The bulk density of the pellets prepared in this manner was approximately 42 lb. per cubic foot. Alternate bales of hay from the uniform supply were run through a field chopper which was set to cut the hay into approximately 3/4-in. lengths. For those treatments involving concentrates, a mixture of the following composition was used: barley, 54%; molasses beet pulp, 20%; wheat mixed feed, 15%; cottonseed meal, 10%, and salt, 1%.

Both forms of hay were fed individually in such weighed amounts as would insure refusals. When concentrates were involved, they were fed according to the requirements of individual cows, based upon the hay consumption and level of production observed during the previous week. Occasionally, particularly toward the end of the trial, certain cows consumed sufficient hay to meet their energy requirements, and in those cases a minimum of 2.0 lb. concentrate was fed daily.

Representative samples of all feeds, fed and refused, were taken for the determination of dry matter. Milk produced was weighed at each milking and sampled for daily determinations of butterfat by the Babcock procedure. Each cow was weighed three days in succession at the beginning and at the end of each experimental period. All observations were based on 28-day periods, preceded by seven-day preliminary periods in the double reversal scheme.

RESULTS AND DISCUSSION

The results of this study are summarized in Table 1. Significantly more 4% FCM ($P < 0.01$) was produced on pelleted than on chopped alfalfa hay when neither form of hay was supplemented with concentrate. Supplementation of the chopped hay with concentrates at the rate of about 12% of the total dry matter intake resulted in a significant increase in production ($P < 0.05$). The

TABLE 1

Response of cows to pelleted and chopped alfalfa hay when fed with and without concentrates

	Pelleted hay	Chopped hay	Pelleted hay plus concentrate	Chopped hay plus concentrate
Daily 4% FCM *(lb.)*	28.1	23.7	27.9	27.0
Ave. B.F. *(%)*	4.01	4.09	3.93	3.97
Daily D.M. intake *(lb.)*	33.3	26.4	35.4	31.1
Concentrate *(% of total D.M.)*			12.1	12.2
Live weight gain per period *(lb.)*	46.7	−3.3	35.0	11.5

addition of a similar amount of concentrate to the pelleted hay ration had no significant effect upon production, so that there was no difference in the cows' performance between pelleted and chopped hay when each was supplemented with concentrates.

The levels of production in this trial seemed to depend on the levels of dry matter intake. Thus, when the two forms of hay were fed without concentrates, the pelleted hay resulted in a significantly higher ($P < 0.05$) dry matter intake than the chopped hay. When chopped hay was supplemented with concentrates, the dry matter intake was increased by an amount which was nearly significant at the 5% level ($P \simeq 0.07$), such that the daily dry matter consumed was not different from that consumed from either of the rations containing pellets.

The butterfat percentage of the milk produced was not affected by the feeding of pelleted hay in this study. It should be observed that the rate of roughage intake was not greatly depressed when the relatively low levels of concentrate were added to the all-hay rations. The ground roughage rations which Powell (7) implicated as being fat depressing involved relatively low intakes of hay. In work conducted at Connecticut (5), a depression of butterfat percentage was observed on a pelleted hay ration when hay consumption decreased about 30%. These observations suggest that any effect upon the butterfat percentage may not be due to the fine grind per se but in interaction with the level of hay intake and/or concentrate intake. Thus, the possibility exists that the depression of the level of intake of ground and pelleted hay, due to relatively high rates of concentrate feeding, may have an effect on the butterfat percentage. Certain field observations, also, have suggested this possibility.

Evidence has been offered (1, 2) which indicates that the type of concentrate fed in conjunction with ground and pelleted hay has an effect on the butterfat content of milk. The effect appears to be associated with changes in the proportions of rumen volatile fatty acids. Earlier, Tyznik and Allen (10) had shown that low roughage intakes caused similar rumen VFA changes with corresponding decreases in the butterfat percentage of milk.

The loss of live weight observed when cows were fed solely on chopped hay was probably due largely to a loss in body fill associated with the lower dry matter intake. The average live weight changes of the cows receiving only pelleted and only chopped hay were significantly different.

No real difficulty was experienced with respect to the cows' acceptability of the pelleted hay. Ration changes were made abruptly and in only one case was it necessary to coax a cow to accept the pellets and then only during the first three days of offering.

ACKNOWLEDGMENT

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RESPONSES OF YOUNG CALVES TO A DIET CONTAINING SALTS OF VOLATILE FATTY ACIDS[1, 2]

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SUMMARY

Nine calves, starting at five days of age, were fed the following diets 12 wk.: Diet I, casein, fat, glucose, starch, and chopped hay; Diet II, casein, fat, glucose, and salts of acetic, propionic, and butyric acids, and Diet III, whole milk. For these diets, the average daily gains during the terminal 9 wk. were 0.82, 0.99, and 1.38 lb., respectively. After feeding, blood glucose increased sharply for Diet III but decreased slightly for Diets I and II. Conversely, blood volatile fatty acids (VFA) increased markedly for Diets I and II, but did not change for III. Increases of blood ketone bodies after feeding were somewhat more pronounced for Diets I and II than for III. Only small differences among diets were noted for any of the blood constituents immediately before feeding. At 0-hour, glucose tended to decrease with increasing age, whereas VFA and ketone bodies exhibited no marked change. The quantities of urinary ketones and VFA were largest for calves fed Diet II, whereas the quantities of urinary glucose were greatest for calves receiving Diet III. In general, the total urinary excretion of all these constituents tended to increase with advancing age. The responses indicate that calves at 3 wk. of age definitely are able to absorb VFA.

The presence of volatile fatty acids (VFA), primarily acetic, propionic, and butyric (17), among microbial fermentation end-products in the rumen is one of the distinguishing characteristics of functional development of this organ. Advent of this fermentation in young ruminants subjected to the usual husbandry regimen apparently is a gradual and progressive process. The extent to which exposure of rumen epithelium to the fermentation environment is necessary to effect fatty acid absorption has not been elucidated adequately. Recent development of a purified type of fatty-acid diet (13) that ruminants will consume at an early age provided a means of further ascertaining whether or not the young calf can utilize volatile fatty acids from a rumen that has not undergone a normal anatomical and functional evolution. Thus, the purpose of this investigation was to gain, by means of this fatty acid diet, additional information on VFA production and utilization in young ruminants.

EXPERIMENTAL PROCEDURES

Dietary treatments were as shown in Table 1. Responses of calves fed the volatile-fatty-acid diet (II) described by Matrone *et al.* (13) were compared with responses to two control diets, a starch chopped-hay diet (I), and a whole-milk

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[2] The data in this paper are from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science.

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TABLE 1
Composition of experimental diets

Ingredients	Amounts per 100 lb. of diet		
	Diet I	Diet II	Diet III
	(lb.)		
Casein	30.0	30.0	.....
Hydrogenated vegetable oil[a]	4.0	4.0	.....
Glucose[b]	24.1	24.1	.....
Corn starch	21.9	.....	.....
Chopped fescue hay	10.0	.....	.....
Sodium acetate[c]	.....	9.0	.....
Potassium acetate	.....	8.5	.....
Magnesium acetate	.....	3.0	.....
Sodium propionate[d]	.....	3.0	.....
Calcium propionate[d]	.....	4.8	.....
Sodium butyrate[c]	.....	3.6	.....
Mineral mixture	5.0[e]	5.0[f]	[g]
Vitamin mixture	5.0[h, i]	5.0[h, i]	[i]
Whole milk[j]	....	.....	100.0

[a] Vegetable shortening supplied through the courtesy of Buckeye Cotton Oil Co., Cincinnati, Ohio.

[b] Cerelose, Corn Products Sales Co., Norfolk, Va.

[c] Contributed by the Farmers Cooperative Exchange, Raleigh, N. C.

[d] Contributed by E. I. duPont de Nemours and Co., Wilmington, Del.

[e] $CaHPO_4$, 818 g.; KCl, 273 g.; NaCl, 239 g.; $MgSO_4$, 204 g.; $CuSO_4 \cdot 5H_2O$, 893 mg.; $FeSO_4 \cdot 2H_2O$, 7,648 mg.; $MnSO_4 \cdot H_2O$, 1,399 mg.; ZnO, 2,263 mg.; $CoCO_3$, 9.2 mg., and glucose, 722 g.

[f] Cu, Fe, Mn, Zn, and Co salts, same as in [e]; NH_4Cl, 138 g.; H_3PO_4 (85%), 226 g., and glucose, 1,892 g.

[g] $CuSO_4 \cdot 5\ H_2O$, 120 mg.; $FeSO_4 \cdot 7\ H_2O$, 763 mg.; $CoCO_3$, 1.2 mg.; $MgSO_4 \cdot 7\ H_2O$, 18.54 g., and $MnSO_4 \cdot H_2O$, 188 mg.

[h] Thiamine HCl, 400 mg.; riboflavin, 850 mg.; nicotinic acid, 1.13 g.; Ca pantothenate, 1.42 g.; pyridoxine HCl, 570 mg.; folic acid, 57 mg.; *p*-aminobenzoic acid, 1.13 g.; inositol, 11.35 g.; biotin, 11 mg.; choline chloride, 113.45 g.; menadione, 115 mg.; 0.1% B_{12} in mannitol, 4.66 g.; alpha-tocopherol acetate, 570 mg., and glucose, 2,132 g. Biotin contributed by Hoffmann-LaRoche, Nutley, N. J., and other vitamins by Merck and Co., Rahway, N. J.

[i] Vitamins A and D were given to all calves via capsule at the daily rate of 10,000 and 1,000 I.U., respectively. Contributed by R. P. Scherer Corp., Detroit, Michigan.

[j] Fresh whole milk testing between 3.3 and 3.7% fat.

diet (III). Diet I was a purified type modified by including chopped hay, in order that its properties would correspond, at least in part, to a roughage diet. Diet II, supplying acetic, propionic, and butyric acids in the same proportions as they usually occur in a normally functioning rumen (16, 17), was similar to Diet I, except that salts of these acids were used in lieu of hay and starch. Diet III, whole milk, was included because it is the normal preruminant food of young calves.

The experimental subjects were nine male Holstein calves, which remained with their respective dams from birth to two or three days of age. Shortly thereafter, they were assigned to three replications on the basis of the time that they became available. Calves within replications were allotted to the diets at random. A 12-wk. experimental period was subdivided into four 3-wk. phases, the first of which was utilized for dietary adjustments. Calves of all groups received whole milk, via nipple pails, at the daily rate of 10 lb. per 100 lb. of body weight from the age of two or three days to 2 wk. During the following week, the amounts of milk given to animals of dietary Groups I and II were reduced gradually to effect a complete transition to the respective solids experimental diets, which were

introduced when the calves were five days of age. Calves of dietary Group III, restricted to whole milk, continued at the initial rate of intake throughout the remainder of the experimental period. Housing quarters were individual pens free of bedding. Water was accessible at all times, but feeds were offered only twice daily.

Criteria of response to the dietary treatments included water and feed consumption, weight gains, general health, changes in concentrations of VFA, total reducing substances (hereafter referred to as glucose), and ketone bodies in the blood, and quantities of thees constituents excreted in the urine. At weekly intervals throughout the experiment, blood samples were drawn from the jugular vein immediately prior to feeding and thereafter at 3 and 6 hr. A longer series of blood samples was collected at 3 and 12 wk. of age, starting immediately before feeding and continuing at 1, 2, 4, 6, 8, 10, and 12 hr. postfeeding. Potassium oxalate was used as the anticoagulant. A 24-hr. collection of urine was obtained at weekly intervals by transferring the calves to metabolism crates. Toluene was used as a preservative for the urine.

Samples of blood and of urine were analyzed for VFA by the steam-distillation method of McClendon (11), for glucose by the method of Nelson (15), and for ketone bodies by the method of Greenberg and Lester (6). Values for VFA and ketone bodies were expressed as acetic acid and acetone, respectively. Gross energy of the dry diets, I and II, was determined by means of a Parr bomb calorimeter and that of the milk diet, III, was calculated from its average content of fat and its estimated content of protein and lactose.

RESULTS

Intake and gains. As indicated in Table 2, calves receiving Diet II, containing salts of VFA, consumed almost twice as much water as did calves eating the other dry diet, I. Intake of supplemental water by calves given Diet III was exceedingly low.

Gross energy intake, weight gains, and computed efficiency of gross energy utilization (Table 2) were similar for dietary groups I and II. The gross energy intake of calves fed Diet III was similar to those of the other two groups, yet

TABLE 2

Average daily water and feed consumption and weight gains of the experimental calves[a]

Diet	Consumption			Weight gain	Efficiency of gross energy
	Water[b]	Feed[c]	Gross energy[d]		
	(lb.)	*(lb.)*	*(kcal.)*	*(lb.)*	*(lb. gain/1,000 kcal.)*
I	10.2	2.37	4,300	0.82	0.19
II	22.1	2.50	4,450	0.99	0.22
III	0.7	1.79	4,235	1.38	0.33

[a] Data in this table are from the last 9 wk. of the study only.
[b] Water consumption was recorded for Replicate 3 only.
[c] Consumption of Diets I and II is recorded on an air-dry basis and that of Diet III on an estimated dry weight basis, employing 12.2 as the per cent solids of whole milk.
[d] Kilocalories per pound of diet are as follows: I, 1,813; II, 1,783, and III, 2,615.

the Diet III calves gained more weight ($P < 0.10$) and thus showed a higher efficiency of gross energy utilization ($P < 0.05$).

General health. All calves remained free of clinical abnormalities. Incidences of diarrhea were low throughout. The relative rating of thriftiness, as judged by general appearance, at the end of the experiment, though not markedly different among the dietary groups, was in the following descending order: III, II, and I. Calves in this last group, in contrast with others, frequently bloated slightly after feeding and occasionally ruminated. All calves after 4 wk. of age manifested some degree of pica.

Effect of age and of diet on changes in blood constituents, 6 hr. postfeeding. Data on levels of VFA, glucose, and ketone bodies in samples of blood from calves fed the different diets are presented by successive 3-wk. periods (Figure 1).

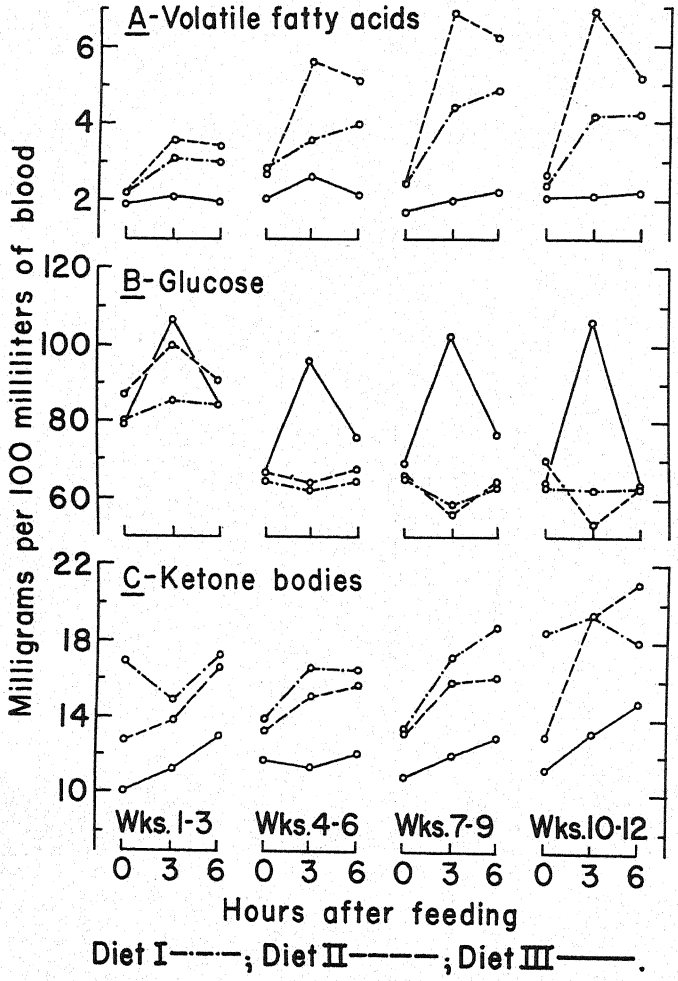


FIG. 1. Effect of age, by 3-wk. periods, and dietary regime on changes in concentrations of volatile fatty acids (as acetic), glucose, and ketone bodies (as acetone) of blood collected from calves at different stages in relation to time of feeding.

Comparative effects of diets on the blood constituents at different ages of the calves are indicated at the prefeeding stage, when absorption during the interim between feedings presumably was at a minimum, and at two post-feeding periods.

a. *VFA.* As shown in Part A of Figure 1, prefeeding concentrations of blood VFA were similar, irrespective of diet and age of animal. After feeding, however, marked increases were noted for Diets I and II, the latter effecting the greater response. The magnitude of these increases tended to become greater with advancing age of the calves. Following the first 3-wk. period, the increases from both Diets I and II showed highly significant deviations from a linear trend ($P < 0.01$), which suggests that the absorption peaks probably were reached either before or shortly after the 6-hr. sampling. The postfeeding concentrations of VFA in the blood of calves fed Diet III were similar to the prefeeding values, which were low throughout the experiment.

b. *Glucose.* Within each of the 3-wk. periods, prefeeding concentrations of glucose were about the same for all dietary groups (Part B, Figure 1). From the first period through the second, however, the values for the prefeeding samples decreased, after which the concentrations tended to plateau. Such values declined from approximately 90 mg. per 100 ml. of blood during the 1st week to about 65 mg. by the end of the 6th wk. Distinct postfeeding increases in blood glucose were noted only when milk was in the diet, the highest level being at the 3rd hr., followed by a precipitous decrease by the 6th hr. The peak values from Diet III tended to increase in succeeding periods after the 4–6 wk. period. The postfeeding blood-glucose patterns from Diets I and II tended to be antithetical to the pattern from Diet III.

c. *Ketone bodies.* Presentation of data on ketone bodies in blood (Part C, Figure 1) is restricted to Replication 3, inasmuch as procedural difficulties in the analysis of some samples from earlier replications were encountered. Though there were marked fluctuations in ketone concentrations from week to week, the levels in both pre- and postfeeding samples were higher for calves fed Diets I and II, respectively, than for the calf fed Diet III. Furthermore, the levels from the Diet I calf tended to be slightly higher than those from the Diet II calf. Postfeeding concentrations of ketone bodies were greater than the prefeeding levels, the increases from Diets I and II, for the most part, being more pronounced than from Diet III. The pattern of postfeeding increases in successive periods is not distinct, but there was a tendency for the magnitude to increase after the second period.

Effect of diet on changes in blood constituents, 12 hr. postfeeding. Although 12-hr. studies were conducted at both 3 and 12 wk. of age, only the data from the latter are presented (Figure 2), inasmuch as the 3-wk observations included only one replication of calves. The patterns at these two different ages, however, were similar.

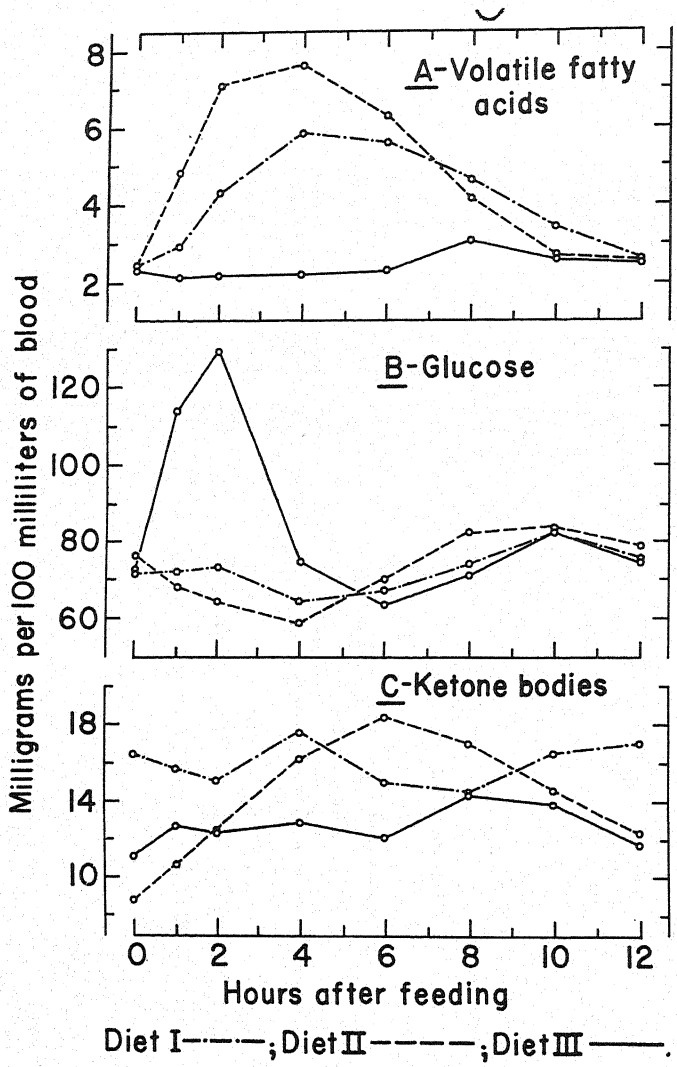


FIG. 2. Effect of dietary regime on changes in concentrations of volatile fatty acids (as acetic), glucose, and ketone bodies (as acetone) of blood collected from calves at 12 wk. of age for 12 hr. postfeeding.

a. *VFA.* The initial trends in the 12-hr. patterns (Part A, Figure 2) were similar to those of the 6-hr. patterns at 10–12 wk. of age (Part A, Figure 1). After ingestion, pronounced increases of VFA were evident in calves receiving Diets I and II. A peak was reached near the 4th hr., after which there was a gradual decline. The terminal values were about the same as the initial. Of these two dietary groups, the rate of increase and the peak concentration were greater for Diet II than for Diet I. In contrast, the postingestion levels of VFA for calves fed Diet III were essentially the same as the preingestion values; the only distinct deviation was a slight rise noted at the 8th hr.

b. *Glucose.* As shown in Part B of Figure 2, the postingestion trends in blood glucose of calves fed Diets I and II were slightly downward during the first 4 hr., followed by a gradual increase to the 10th hr. In contrast to this pattern, the changes for calves fed Diet III were abruptly upward, followed by a precipitous drop to a level slightly below the initial. After the 6th hr., the trend was the same as for the two other groups.

c. *Ketone bodies.* Presentation of data on changes in ketone bodies for 12 hr. postfeeding (Part C, Figure 2) is restricted to Replications 2 and 3, for reasons given previously. The initial postprandial trends for different dietary groups were similar to corresponding trends shown for the terminal 3-wk. period (Part C, Figure 1). The most distinct pattern among the three groups was that for Diet II. The ketone bodies in the blood of this group increased progressively during the first 6 hr., but decreased thereafter. The highest concentrations for dietary Group I were found at the 4th hr. and for Group III at the 8th hr., but these peaks were only small deviations from general trends that tended to remain level throughout the 12 hr. In view of the marked variability in values, no specific pattern was evidenced.

Changes in amounts of various constituents excreted in urine. A summary of observations on the elimination of VFA, glucose, and ketone bodies in the urine is presented in Table 3.

TABLE 3

Mean changes, by age periods, in the urinary excretion of volatile fatty acids, glucose and ketone bodies by calves fed different experimental diets

Urinary constituents	Diet No.	Mean excretion/calf/24 hr. in successive age periods			
		1–3 wk.	4–6 wk.	7–9 wk.	10–12 wk.
		(g.)			
Volatile fatty acids	I	0.22	0.42	0.52	0.95
	II	0.46	1.15	2.19	4.00
	III	0.21	0.26	0.46	0.58
Glucose	I	1.64	0.73	1.47	1.88
	II	1.62	1.19	2.73	5.55
	III	2.08	1.75	8.60	8.93
Ketone bodies[a]	I	0.35	0.65	0.81	0.87
	II	0.41	1.49	1.54	2.26
	III	0.30	0.40	0.46	0.54

[a] Data from only one replication.

The average daily excretion of VFA in urine increased significantly ($P < 0.01$) as the calves advanced in age. The total amount eliminated, as well as the ratio of increase in excretion, was greatest for the Diet II group. The quantities of VFA in the urine of calves fed Diets I and III were similar.

The 24-hr. excretions of glucose in the urine were similar for all dietary groups through 6 wk. of age. Thereafter, there were increases in amounts eliminated by calves in dietary groups II and III, but no marked change for Group I. However, the differences in excretion by dietary groups during the two terminal periods were not statistically significant.

Excretion of ketone bodies also tended to increase with advancing age of the calves and was greater for Diet II than for either Diet I or III, the values for these two groups being similar.

DISCUSSION

The response of calves to the experimental diets described herein indicates that VFA, either produced in the rumen or ingested in the diet, are absorbed and subsequently metabolized by ruminants as young as 3 to 6 wk. of age. This apparent capacity of the young calf to utilize VFA is in accord with results from studies of rumen metabolism (4, 10) and findings from applied feeding trials (14, 18).

Normalcy of growth of calves receiving the diet containing 10% hay (Diet I), in contrast with poor growth of lambs fed a similar diet without hay (13), indicates that roughage, in accordance with the observations of others (3), plays a beneficial role in overcoming the inadequacies of "purified diets" for ruminants. The effectiveness of supplementary bicarbonates of sodium and potassium in correcting the deficiencies of the roughage-free starch-glucose diet led to the hypothesis that roughage ingestion increases the buffering capacity of the rumen (12). This concept is supported by other results (22), showing that as the amount of hay in the diet of cows was increased, a concomitant rise occurred in pH and in sodium concentration of the rumen contents.

Further interpretation of the metabolism of the experimental diets is based on the assumptions *(a)* that as the ingesta traversed the alimentary tract, the initial repository of the solid materials (Diets I and II) was the reticulo-rumen sac (21), whereas that of the fluid (Diet III) was the abomasum, and *(b)* that concentrations of VFA in the rumen were reflected in changes in the composition of peripheral blood (16). Though postprandial increases of VFA indicate that the calf as young as 3 wk. of age is able to absorb these acids from the rumen, this capacity probably exists at younger stages. However, efficiency of absorption may increase as the morphology of the rumen epithelium changes (2, 23). Since soluble carbohydrates entering the reticulo-rumen cavity are converted to VFA (16), the glycemic increases in milk-fed calves probably reflected primarily absorption from the small intestine. Though Barhydt and Dye (1) postulated that monosaccharide absorption from the small intestine of ruminants decreases with age, this change was not manifested in the magnitude of the alimentary hyperglycemia of calves as they became older.

As the calves fed the solid diets advanced in age, increases in postprandial peak concentrations of blood VFA correspond to observations on calves subjected to a practical type of dietary regime (10). This trend may be attributed to enhancement of efficiency of absorption accompanying rumen maturation, but evidently, increased consumption and augmented production of VFA (4, 10) also played a significant role. The insignificant changes of VFA in the blood of calves restricted to the milk diet indicated relatively little rumen activity.

Since ketone bodies are derived chiefly from the metabolism of fatty acids, absorption of VFA presumably would be related to concentrations of ketone

bodies in the blood and their excretion in the urine. Within the respective dietary groups, the postprandial blood ketone changes beyond the first 3-wk. period tended to follow a pattern similar to that of VFA, but the relationships are not well-defined. The blood ketone levels in the calves receiving the solids diets probably represented a balance between the antiketogenic effects of propionic acid and the ketogenic effects of acetic and butyric acids (5). Among the diets, the order of urinary excretion of ketone bodies did not correspond to the general relative levels of these metabolites in the blood. Since the renal threshold for ketone bodies is low (9), their elimination in urine might have reflected the maximum concentrations in blood, as shown in the 12-hr. studies, to a greater degree than the total production.

Neither age of the calves nor their dietary regimes significantly affected "postabsorptive" levels of blood VFA. During the period of approximately 6 wk. after birth, glucose levels declined, in harmony with findings of others (7, 8, 9, 10, 19, 20), from levels characteristic of simple-stomach species to those typical of mature ruminants. Since this downward trend is negatively correlated with changes in alimentary production of VFA in young ruminants subjected to a milk-concentrate-roughage regimen, an a priori relationship has been suggested (9). This postulation is not supported by the observations *(a)* that postabsorptive changes of concentrations of glucose and of VFA in blood of calves within the various dietary groups are independent and *(b)* that glycemic levels in milk-fed calves declined, even though rumen fermentation was minor. This evidence, in addition to that summarized by McCarthy and Kesler (10) and by Ratcliff *et al.* (19), indicates that declines in the blood glucose of calves following birth are not directly related to the functional development of the reticulo-rumen segment of the stomach. Whenever rumen fermentation is prominently involved, however, the blood glucose patterns presumably are determined, in a measure, by the glucose-depressing tendency of butyric acid and the elevating effect of propionic acid (5).

Higher glycemic levels and lower glucose excretion during the first 6-wk. experimental stage than during the second suggest that the younger calf may have a higher renal threshold than the older and may be more dependent upon and efficient in the metabolism of glucose. This observation and the aforementioned VFA-glucose relationships in blood during the early stages of the young calf are indicative of basic metabolic changes. The transitional trends are presumptive evidence supporting the hypothesis that the importance of glucose metabolism becomes quantitatively less as the fatty-acid metabolism becomes greater. Although glucose is an indispensable metabolite throughout the life of the ruminant and fatty acids are an important source of energy after the early stages of development, the relative roles of these nutrients at different stages in the physiological development of ruminants have not been clarified adequately.

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EFFECT OF SYNTHALIN A ON RENAL FUNCTION IN DAIRY BULL CALVES AS MEASURED BY PHENOLSULFONPHTHALEIN CLEARANCE [1]

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SUMMARY

Synthalin A (decamethylenediguanidine) is a toxic, hypoglycemic agent which has been reported to cause histological changes in the liver, kidneys, and alpha cells of the Islets of Langerhans, and also to depress hepatic function in dairy bull calves. Five Holstein bull calves (eight days of age) were treated with a single intramuscular injection of synthalin A at the rate of 2.2 mg. per kilogram of body weight. Renal function as measured by both phenolsulfonphthalein fractional and volume clearances was depressed to approximately 35% of normal within 16 hr., and had returned to near-normal values within seven days. Correspondingly, plasma glucose levels decreased from a mean preinjection level of 85.6 mg. % to 32.8 mg. % at 16 hr. after injection, and returned to normal levels again within 66 hr. after injection. These effects were significant statistically ($P < 0.01$).

Synthalin A (decamethylenediguanidine) was synthesized in 1926 by German investigators (7) who were attempting to produce an orally active substitute for insulin. The material was effective in controlling the hyperglycemia of diabetics, but its use was discontinued because of its severe toxicity.

Several workers (4, 5, 6) have shown that synthalin A caused degenerative changes in the alpha cells of the Islets of Langerhans, the presumed source of a hyperglycemic-glycogenolytic factor, glucagon.

Richards and Weaver (13) and Mixner *et al.* (12) have shown that synthalin A is a hypoglycemic agent in dairy cattle. Mixner *et al.* (12), in addition, reported a decrease in liver function as measured by sulphobromophthalein (BSP) fractional clearance.

Blatherwick *et al.* (3) reported that synthalin A in rabbits caused extensive damage to the cells of the convoluted tubules but not to the glomeruli of the renal nephrons. Davis (4), also working with synthalin A in rabbits, found a severe necrosis of the proximal convoluted tubules of the renal nephrons, along with evidence of liver damage.

The purpose of this investigation was to determine if synthalin A has a detrimental effect on renal function in dairy bull calves as measured by phenolsulfonphthalein clearance (11) and, if so, to implicate the damaged kidney as another causative factor in the hypoglycemic action of synthalin A. A brief report has been made on this study (1).

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METHODS AND PROCEDURES

Five Holstein bull calves were used in this experiment, each animal serving as its own control. Renal function was determined in these animals, using phenolsulfonphthalein (PSP) clearance measures (2, 11).

A single intramuscular injection of synthalin A was given each animal at the rate of 2.2 mg. per kilogram of body weight at 5 P.M. on the eighth postnatal day. The PSP clearance tests were performed on each animal on the seventh, eighth, ninth, and 16th postnatal days between 9 and 10 A.M. Thus, the determinations performed on the seventh and eighth days served as controls, the determination on the ninth day (16 to 17 hr. after synthalin A injection) showed the effects of the synthalin A, and the determination on the 16th day was to show the nature of the recovery.

Blood plasma glucose levels were determined by the glucose oxidase enzyme method (16) on samples collected at 9 A.M. on the seventh, eighth, ninth, tenth, 11th, and 16th days of age.

RESULTS AND DISCUSSION

Mean PSP clearance measures and plasma glucose levels for the five animals are presented in Table 1. An analysis of variance indicated that the PSP fractional clearances and the PSP volume clearances on Day 9 (16 hr. after synthalin A) were significantly lower than the corresponding control values on Days 7 and 8 ($P < 0.01$). The PSP volumes of distribution (per cent of body weight) were not significantly affected by the treatment. PSP fractional clearances and PSP volume clearances (milliliter of plasma per minute per kilogram of body weight) were reduced to 37.4 and 32.2% of normal, respectively (comparison of ninth-day values to mean of seventh- and eighth-day values). Renal function was approaching normal by the 16th day (seven days after treatment).

Plasma glucose levels fell from a mean of 85.6 mg.% on Days 7 and 8 (prior to synthalin A) to 32.8 mg. % 16 hr. after the administration of synthalin A,

TABLE 1

Effect of a single intramuscular injection of synthalin A (2.2 mg. per kilogram) upon plasma glucose and PSP clearance measures of renal function (mean of five bull calves)

Characteristic	Age in days					
	7	8	9	10	11	16
Physical condition	Normal	Normal	16 hr. after synthalin A	Recovery phase		
Body weight *(kg.)*	44.8	45.1	45.2	.....	.....	45.5
Plasma glucose *(mg. %)*	87.2	83.9	32.8	54.3	100.4	84.6
PSP fractional clearance *(per min.)*	0.0257	0.0288	0.0102	.....	.....	0.0220
PSP volume of distribution						
Total *(ml.)*	9,600	9,700	9,300	.....	.....	10,300
Per cent of body weight	23.1	22.0	20.2	.....	.....	23.2
PSP volume clearance						
ml/min	222	268	82	.....	.....	226
ml/min/kg body weight	5.27	6.09	1.83	. .	....	5.07

a significant decline ($P < 0.01$). Plasma glucose levels returned to normal or above within 66 hr. after synthalin A treatment.

All calves showed some outward signs of toxicity to the synthalin A. This was characterized by general lethargy, lassitude, and apathy. However, the animals did continue to eat and move about in their pens. Recovery by outward appearances was rapid, paralleling the return of blood glucose levels to normal.

Phenolsulfonphthalein (PSP or phenol red) was shown by Marshall and Vickers (10) and others (8, 9, 14) to be eliminated from the kidneys primarily by proximal tubule secretion. Since synthalin A causes damage to the proximal tubules (4), it is not surprising that the PSP clearance measures were effective in showing depressed functional activity of these tubules. Similarly, the hypoglycemia caused by synthalin A also may be related to the proximal tubule damage, since the site of glucose reabsorption after glomerular filtration is acknowledged to be the proximal convoluted tubules (15). These data tend to validate the measures of renal function as previously described (2, 11).

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TECHNICAL NOTES

DIRECT ANALYSIS OF LACTOSE IN MILK AND SERUM[1]

Barnett and Tawab (1) showed that the colorimetric phenol-sulfuric acid method of Dubois *et al.* (2) could be used for the direct determination of lactose in milk and cheese. The authors stated that the amount of phenol added was not critical, provided that the same quantity was used throughout. However, variations which occurred in our replicate analyses of standard lactose solutions were traced to minute differences in the amount of phenol reagent added. No information appeared to be available concerning the effect of sulfuric acid concentration on color development. The effect of both reagents on the determination of lactose was, therefore, studied.

Intensity of the color obtained with various concentrations of phenol (Figure 1-A) was maximum between 5 to 12 mg/ml of total arithmetic volume. In comparison, the concentrations used by Barnett and Tawab (1) and Dubois *et al.* (2) were approximately 18.5 and 15.5 mg/ml, respectively; these levels are outside the optimum range and can cause variation in color intensity if not made very accurately. The concentration of sulfuric acid also influenced the intensity of the color (Figure 1-B). Maximum intensity was obtained when the volume of sulfuric acid comprised from 70 to 75% of the total arithmetic volume, a proportion similar to that recommended by previous authors (1, 2).

On the basis of these investigations, we recommend the following procedure, which introduces 11 mg. of phenol and 0.74 ml. of sulfuric acid per milliliter of total arithmetic volume.

PROCEDURE

Pipette 2.0 ml. of test solution (10-75 μg. of lactose), or standard lactose monohydrate solution, or water (for the blank) into a colorimeter tube, followed by 0.10 ml. of 89% w/v phenol reagent (20 ml. water + 80 g. phenol, dissolved at 50° C.; the reagent is stable for at least 4 mo. at room temperature). Add 6.0 ml. of concentrated sulfuric acid slowly, letting the acid run down the side of the tube. Then, swirl to obtain good mixing, let stand for 10 min. at room temperature, and read at 490 mμ (the 470-mμ filter on an Evelyn colorimeter is equally satisfactory). Color is stable for at least 1 hr.

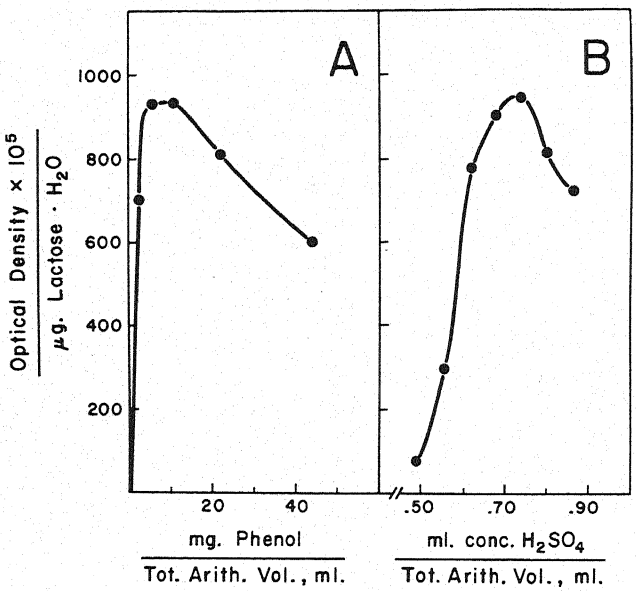


FIG. 1. Effect of concentration of reagents on color intensity. (Total arithmetic volume, 8.1 ml.; readings made at 470 mμ on an Evelyn colorimeter.)

(A) Effect of phenol (0.74 ml. of sulfuric acid/ml).
(B) Effect of sulfuric acid (11 mg. of phenol/ml).

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EFFECTS OF PENICILLIN ON THE MORPHOLOGY OF *STREPTOCOCCUS LACTIS, STREPTOCOCCUS THERMOPHILUS,* and *LEUCONOSTOC DEXTRANICUM* [1]

Numerous reports have appeared in the literature on the effect of penicillin upon the growth and acid production of lactic acid bacteria. Inhibition of growth or of acid production by penicillin in lactic cultures is the basis for tests that have been developed to detect penicillin and other antibiotics in the milk supply. Reviews by Overby (7), by Stoltz and Hankinson (9), and by Berriidge (3) cover the subject thoroughly.

Werber-Alture *et al.* (10) and Pulvertaft (8) reported that penicillin, in low concentrations, caused morphological changes in *Escherichia coli* cultures. Chain *et al.* (4) observed this same reaction using *Staphylococcus* cultures. Gardner (5) observed morphological changes caused by penicillin in a variety of cultures. Baughman and Nelson (2) reported similar results with some lactic cultures after this study was in progress.

This investigation was made to determine the effect of penicillin on the morphology of certain lactic acid bacteria.

EXPERIMENTAL PROCEDURE

Cultures of *Streptococcus lactis, Streptococcus thermophilus,* and *Leuconostoc dextranicum,* obtained from the Department of Bacteriology, University of Wisconsin, Madison, were used in the study. The cultures were grown in 10% sterile reconstituted nonfat dry milk and transferred in the usual manner. *S. lactis* and *S. thermophilus* were used for inoculum after incubation at 37° C. for 8–10 hr.; *L. dextranicum* after incubation for 16 hr. at 25° C.

Penicillin (G Potassium)[2] was prepared in sterile buffered aqueous solution, dispensed in 1-ml. portions (1,000 units) in sterile test tubes and immediately placed in storage at — 15° C., until needed for the tests.

When removed from storage, the 1-ml. portions of penicillin were thawed at room temperature and diluted 1:1,000 in sterile buffered distilled water just prior to use. The 1:100 and 1:1,000 dilutions were dispensed into sterile test tubes to give final concentrations in a 10-ml. volume of 0.025, 0.05, 0.10, 0.20, and 0.50 units/ml. A control containing no penicillin was prepared in each series of tests. Sufficient sterilized antibiotic-free skimmilk (10% reconstituted NDM) was added to bring the volume of the mixture up to 9.5 ml. in each tube. Next, 0.5 ml. of the test culture was added per tube, bringing the total volume in each tube to 10 ml. Mixing of the contents was accomplished by inverting the tubes three times. Methylene blue stains (1) were made on each test after 30, 45, 60, and 120 min. incubation at 32 and 37° C. for *S. lactis* and *S. thermophilus;* for *L. dextranicum,* stains were made after 4, 8, and 16 hr. incubation at 25° C. These stains were examined with a microscope, using oil immersion, and compared to the control to observe changes in morphology.

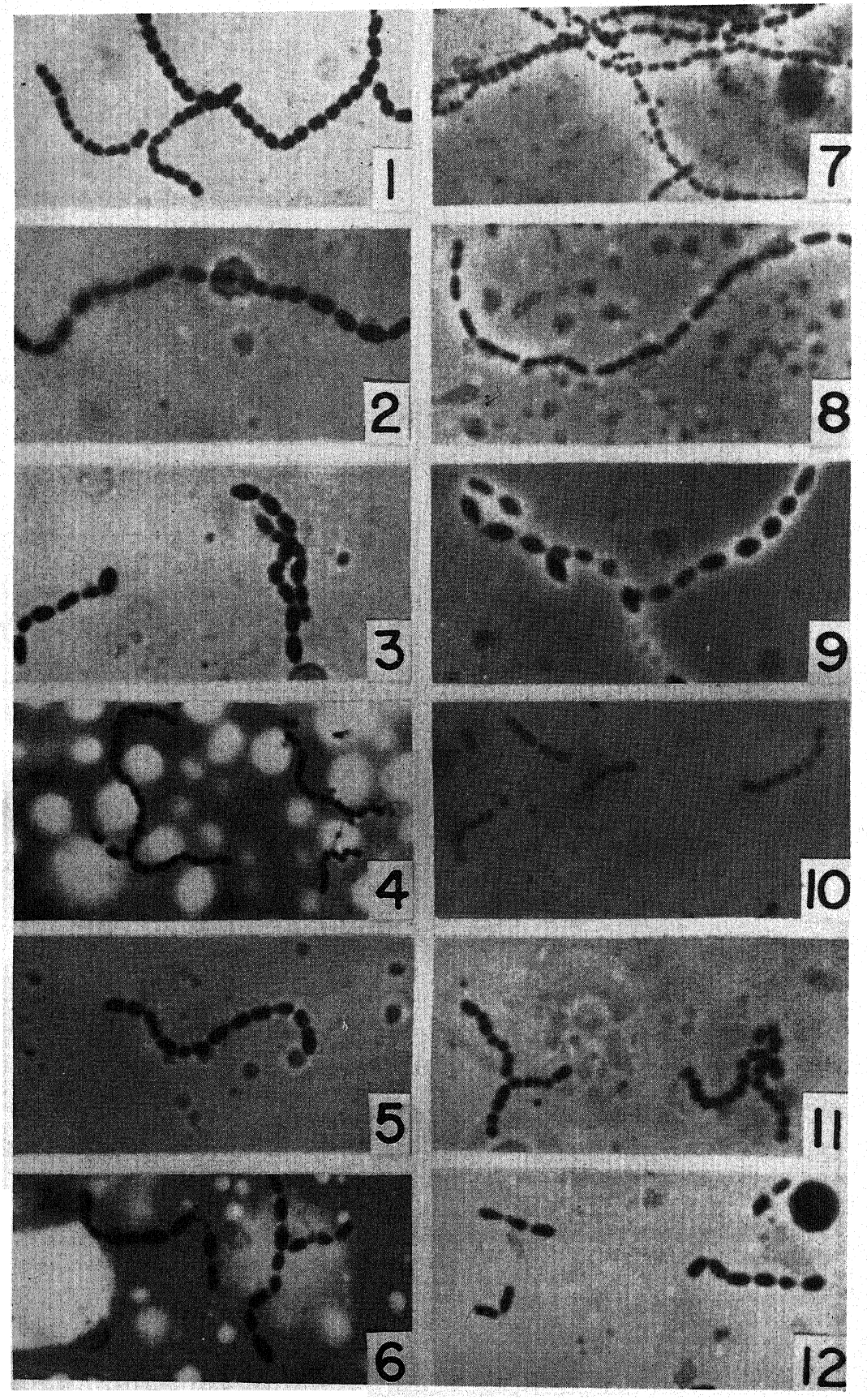
Two cows of the Agricultural Experiment Station herd were given, in each quarter, infusions of an antibiotic mixture containing 100,000 units of penicillin G, 250 mg. dihydrostreptomycin, 250 mg. sulfisoxazole, 750 mg. sulfathiazole, and 5 mg. cobalt sulfate suspended in 3% aluminum monostearate.[3] Samples were collected from each cow in sterile containers for six consecutive milkings spaced at 12-hr. intervals.

These samples were tested for effects on the morphology of a *S. thermophilus* test culture. For each sample, tests were performed with the milk as obtained and after dilutions with antibiotic-free raw milk of 1:10, 1:100, 1:1,000, and 1:10,000. This procedure gave an indication of the concentration of antibiotics in milk from the various milkings. In the test, 9.5 ml. of a sample (as drawn or as diluted) were placed in a sterile test tube to which 0.5 ml. of culture was added, tubes were inverted to mix contents, and were placed in a 37° C. bath for 1 hr. Methylene blue stains were prepared for examination and compared with those of a control test with antibiotic-free milk.

[1] Florida Agricultural Experiment Station Journal Series No. 875.

[2] Chas. Pfizer and Company, New York.

[3] Fort Dodge Laboratories, Fort Dodge, Iowa.


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RESULTS AND DISCUSSION

The effects of penicillin on the morphology of three lactic cultures are shown in Figure 1. The main changes in morphology recognizable when using a methylene blue stain are swelled and elongated cells. A similar observation was made by Gardner (5) on other cocci. Apparently, the penicillin in some way inhibits a step in cell division, but the cell continues to grow in size, resulting in the swelled appearance. This is supported by the findings of Chain *et al.* (4), that penicillin allowed one division to take place if the cells were growing rapidly. Cultures used for inoculum in this study were in a rapid growth phase, to take advantage of this factor.

Pulvertaft (8) reported that aureomycin, streptomycin, and chloromycetin caused changes in morphology similar to those caused by penicillin, but more time was required for these changes to occur. As penicillin was the fastest-acting antibiotic, and the one most prevalent in the milk supply, it was chosen for a more complete study.

George and Pandalai (6) reported that when using a Gram stain, some reversal in staining characteristics of bacteria was caused by sublethal concentrations of penicillin. However, as enlargement of cells was the first evidence of change, and because the methylene blue stain was simpler to prepare and showed the swelled and enlarged conditions, it was chosen for this work.

The photomicrographs in Figure 1 are typical examples of changes in morphology of the three cultures in the presence of various concentrations of penicillin. As the concentration of penicillin was increased, the swelling and elongation were more pronounced in all cultures used. Further, it appeared that individual cells which had undergone swelling and elongation stained darker than cells not affected by the penicillin.

The results obtained on testing the effects of milk from antibiotic-treated cows on the test culture appear in Figure 1. Pictures 5 and 6 in Figure 1 represent the typical results obtained with the test culture grown in milk produced by a cow that had been treated with an antibiotic mixture by the infusion method.

SUMMARY

The effects of penicillin on the morphology of *Streptococcus lactis, Streptococcus thermophilus,* and *Leuconostoc dextranicum* were mainly a swelling and an elongation of individual cells. Using a methylene blue stain, these changes could be detected with *S. thermophilus* after exposure to concentrations of 0.025 to 0.5 μ/ml of penicillin for 30 min. at 37° C. Results revealed that *L. dextranicum* was more resistant to penicillin than was either *S. lactis* or *S. thermophilus*. Milk from cows treated with a mixture of penicillin and other antibiotics by udder infusion caused morphological changes in the test culture.

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LEGEND FOR FIGURE 1, PAGE 1392

Fig. 1. Methylene blue stains showing morphological changes in various lactic cultures caused by penicillin.[a, b]

1. *S. thermophilus* control; 2. *S. thermophilus* 0.02 μ/ml penicillin; 3. *S. thermophilus* 0.5 μ/ml penicillin; 4. *S. thermophilus* control; 5. *S. thermophilus* milk from treated cow (first milking diluted 1:1,000); 6. *S. thermophilus* milk from treated cow (fifth milking, no dilution); 7. *L. dextranicum* control; 8. *L. dextranicum* 0.2 μ/ml penicillin; 9. *L. dextranicum* 0.5 μ/ml penicillin; 10. *S. lactis* control; 11. *S. lactis* 0.05 μ/ml penicillin; 12. *S. lactis* 0.5 μ/ml penicillin.

[a] Strains of *S. thermophilus* and *S. lactis* after 1 hr. exposure at 37° C. Those of *L. dextranicum* after 16 hr. exposure at 25° C.

[b] Magnification, 1,350 ×.

EFFECT ON SILAGE OF CHOPPING AND BRUISING THE FORAGE[1]

A study was begun in 1958 in which the value of various forages for silage was estimated in 1-qt. jar silos. During the course of this work it was found that the quality of silage produced could be markedly improved by finer chopping and/or grinding, even though the extent of air exclusion and density were constant. This report consists of the preliminary results of a study of the conditions under which the same factor might influence the silage obtained in larger silos.

Two harvesting methods were used which were intended to provide extremes of conditions for physical treatment and allow observations of their effects, if any. The harvesting methods were: 1. Chopped with a forage harvester set for a 3-in. cut. 2. Chopped with a harvester set set for a 5/16-in. cut and this followed by the bruising provided by a Silorator. This reduced the forage to an almost pulpy consistency and is referred to as "bruised." No wilting was involved with either method.

One load of third-cutting alfalfa was harvested by each of the two methods in the morning of September 3 and again in the afternoon. Two 4- by 8-ft. steel silos were filled from each load by a uniform procedure consisting of tramping, weighting with 500 lb. of brick, and sealing with a steel cover and rubber gasket. Thus, four silos were filled from each of the two types of forage.

The silages were opened after a storage period of 145 days and the procedures evaluated on the basis of preservation efficiency and chemical quality of the silage.

The data in Table 1 indicate that pre-ensiling mechanical treatment had a marked effect on final quality of the silages. The bruised silages were characterized by lower pH values, lower ammoniacal nitrogen, and higher lactic acid contents than the chopped silages. Bruised silages also had a much lower butyric acid content in the P.M.–harvested silages. The value of the bruising treatment for improving chemical quality was more pronounced in the silage from the P.M.-harvested forage, since this was the forage which produced the lowest quality silage when chopping was the only treatment.

The final distribution of stored dry matter is presented in Table 2. The assumption has been made that dry matter not recovered as silage or seepage was lost in a gaseous form. Bruised silage consistently had much lower gaseous losses than the chopped silage, although this advantage in the A.M.-harvested forage was counterbalanced by greater seepage losses.

An adequate explanation of the preliminary results of this study is not within the scope of this report if, indeed, possible from the limited data. Apparently, the rupture of a high percentage of the plant cells (bruised) was directly or indirectly responsible for producing a fer-

[1] Associated with graduate studies, undertaken by C. H. Gordon at the University of Maryland, Agronomy Department, College Park, Maryland.

TABLE 1
Final chemical quality of silages

Silo No.	Dry matter	pH	Organic acids: Ammonia N[a]	Butyric	Propionic	Acetic	Lactic
	(%)		(% dry matter)				
A.M.—stored			Bruised				
3	18.3	4.2	1.29	.36	.07	2.83	8.00
10	18.2	4.2	1.30	.06	1.21	2.12	7.28
			Chopped				
1	18.2	4.6	2.08	.06	.04	5.12	4.00
8	17.6	4.6	2.20	.74	.22	4.66	3.11
P.M.—stored			Bruised				
7	21.5	4.0	1.05	.12	.07	1.53	8.55
4	22.5	3.9	1.08	.05	.01	1.45	8.26
			Chopped				
6	20.1	5.0	4.20	4.53	0.55	2.56	0.66
2	20.6	5.2	4.04	4.65	0.12	2.29	0.28
Averages							
Bruised	20.1	4.1	1.18	.15	.34	1.98	8.02
Chopped	19.1	4.8	3.13	2.50	.23	3.66	2.01

[a] Expressed as protein.

TABLE 2
Initial and final dry matter distribution

	Silo No.	Initially stored	Final distribution as % of stored: Silage	Seepage	Gas
		(lb.)			
A.M.—stored			Bruised		
	3	502.2	89.5	7.3	3.2
	10	519.4	93.2	7.2	—0.4
			Chopped		
	1	326.1	88.9	2.5	8.6
	8	357.4	86.1	3.0	10.9
P.M.—stored			Bruised		
	7	506.0	97.9	0.7	1.4
	4	538.4	98.5	2.1	—0.6
			Chopped		
	6	346.2	82.0	0.3	17.7
	2	376.7	84.3	0.3	15.4
Averages					
Bruised		516.5	94.8	4.3	0.9
Chopped		351.6	85.3	1.5	13.2

mentation which was profoundly different from that occurring when the crop was only chopped. Since spoilage was prevented in all silos, oxygen appears to have been well excluded under both treatments. Although density differed with treatments in this experiment, previous work with 1-qt. silos indicates that this was not primarily responsible for the differences observed.

The mechanisms of the effect observed remain obscure, but the results clearly indicate that mechanical preparation of the forage previous to ensiling may be a powerful tool for influencing the quantitative and qualitative characteristics of a silage. The magnitude of differences observed suggests that this tool may be of practical as well as fundamental interest.

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FRACTIONATION OF MILK PROTEINS ON ANION-EXCHANGE CELLULOSE [1]

A method of column chromatography using anion-exchange cellulose was developed by Sober and Peterson (1, 3) and successfully applied to various proteins, nucleic acids, and enzymes. They reported that the resolving power of the cellulose ion-exchanger is greater than that afforded by conventional electrophoresis. They recovered 50–100% of the activity of various enzymes and achieved quantitative nitrogen recovery (3).

An attempt was made to apply this technique in fractionating milk proteins. The procedure used was that described by Sober *et al.* (2) for preparing the adsorbent column and developing the chromatogram.

Freshly drawn, uncooled milk from a single cow was separated into cream and skimmilk immediately after milking, and cooled rapidly with ice water. The skimmilk was dialyzed for 24 hr. against large amounts of the starting buffer (0.005 *M* sodium phosphate at pH 7.0) and the buffer was changed frequently. Twenty-five milliliters of the dialyzed skimmilk was placed on the column (anion-exchange adsorbents DEAE-SF[2], Diethylaminoethanol on Solfa-Floc cellulose lattice) and followed with continuous flow of buffer. Gradients of salt or pH or both were employed for elution. Toluene-covered sodium phosphate buffers were used at flow rates of about 50 ml. per hour (7 p.s.i. N_2). The elution diagram obtained is shown in Figure 1. The individual effluent fractions of about 5 ml. each were examined in a Beckman DU spectrophotometer at 280 mμ.

This simple continuous fractionation by anion exchanger is proving very useful for isolation, purification, and characterization of milk proteins and enzymes. The procedure used may be modified according to the particular components under investigation. A modification of this method is being used in this laboratory for the study of milk lipase systems.

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[2] Obtained from the Bio-Rad Laboratories, Richmond, California.

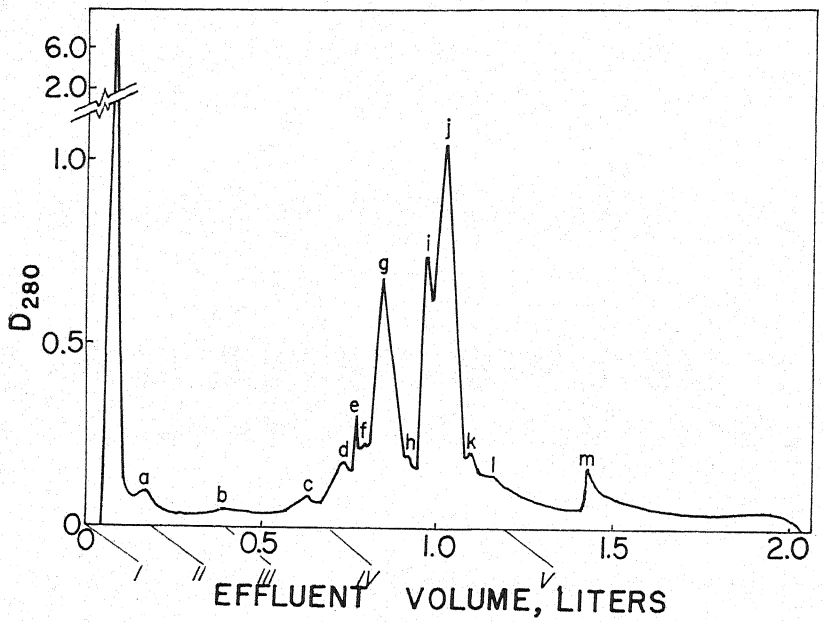


FIG. 1. Effluent diagram of milk protein on DEAE-SF (1.3 × 33 cm.); effluent collected in 5-ml. fractions. Buffers: I, 0.005 *M* Na phosphate, pH 7.0; II, gradient to 0.02 *M* Na phosphate, pH 6.0; III, gradient to 0.05 *M* NaH_2PO_4; IV, gradient to 0.1 *M* NaCl—0.05 *M* NaH_2PO_4; V, gradient to 0.5 *M* NaCl—0.1 *M* NaH_2PO_4.

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CEREBROSPINAL FLUID COMPOSITION STUDIES IN CALVES ON VARYING CAROTENE INTAKES[1]

An increase in cerebrospinal fluid (CSF) pressure appears to be the first measurable change in vitamin A deficiency in the calf (8, 10). On this basis, it appeared plausible that concentration changes of certain constituents in CSF might occur prior to this increased pressure.

Twelve Guernsey and 12 Holstein 64-day old male calves were placed on a vitamin A depletion ration until their blood plasma vitamin A level had decreased to ≤12 γ%. Thereafter, with restrictions as to breed, each animal received one of four carotene intakes, 30, 60, 120, or 240 γ/lb live weight/day, from artificially dehydrated alfalfa. A more complete description of the depletion ration and the experimental procedure used in this study has been reported by Eaton *et al.* (5). The 30-γ intake level was chosen on the basis that it should result in an increased CSF pressure in the Guernsey calves, but be just adequate to prevent this in the Holsteins (9). The remaining levels of carotene were considered more than adequate to prevent an increased CSF pressure in both breeds.

After 12 wk. on their respective rations, CSF pressures were obtained by puncture of the spinal canal through the dorsal opening of the atlanto-occipital articulation and by measuring the height to which the CSF rose, in a rigid polyethylene tube attached to the hub of the needle (7, 11). After measuring CSF pressures, approximately 25 ml. of fluid were withdrawn by means of a hypodermic syringe. Sodium, potassium, and calcium concentrations of CSF

[1] The author is indebted to H. D. Eaton for helpful suggestions in the preparation of this manuscript.

were determined flame photometrically (1, 4), and inorganic phosphorus was measured colorimetrically (6). Analysis for free amino acids in CSF was carried out as follows: Approximately 15 ml. of CSF were evaporated to dryness on the steam bath, to precipitate the proteins, the residue taken up in hot water and the solution filtered. The filtrate was then desalted electrolytically, evaporated to dryness again, and the residue taken up in a small volume of 10% isopropyl alcohol. This solution was used for spotting the chromatograms. Two dimensional paper chromatography was used to separate and identify the free amino acids; 80% pyridine and ethanol : butanol : formic acid : water (60 : 20 : 5 : 15) were the primary solvents employed. Statistical procedures used for the analysis of sodium, potassium, calcium, and phosphorus data were taken from Cochran and Cox (3).

Inspection of the mineral analyses results, substantiated by statistical analysis, revealed that there were inappreciable differences between carotene intake or breed in the sodium, potassium, inorganic phosphorus, and calcium concentrations of CSF (Table 1). Only two calves, both Guernseys on the 30 γ carotene intake group, were found to have an increased terminal pressure (av. 125 mm. H_2O). The present results thus would indicate that no changes in the concentration of these constituents occur prior to an increased CSF pressure; however, the possibility that this balance is altered in a more severe or prolonged deficiency still exists. The only previous report on bovine CSF found in the literature gave a mean calcium value of 5.5 mg/100 ml. (2), which is in reasonable agreement with the present results.

The free amino acids and their per cent occurrence, based on the analysis of CSF from eight Guernsey and two Holstein calves, are given in Table 2. Differences in the per cent occurrence of the free amino acids appeared not to be related to carotene intake or elevated CSF pressure; however, because of the limited numbers (calves) and unequal distribution among carotene intakes and breeds, no definite conclusions could be made from these data. The amino acids reported in Table 2 represent those constituents which were identified; however, several unknown ninhydrin-positive spots were observed in some of the CSF samples. On the basis of visual color density and area of spots, alanine, serine, and glutamine were the amino acids consistently present in the highest concentration.

TABLE 2

Free amino acids present in cerebrospinal fluid of eight Guernsey and two Holstein calves

Amino Acid	Occurrence
	(%)
Alanine	100
Arginine-Lysine	50
Aspartic Acid	40
Cysteine-Cystine	80
Glutamic Acid	100
Glutamine	100
Glycine	100
Leucines	40
Serine	100
Threonine	100
Valine	30

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TABLE 1

Concentrations of sodium, potassium, inorganic phosphorus, and calcium in cerebrospinal fluid of Guernsey and Holstein calves

	Carotene intake[a]					Standard deviation per calf
	30 γ	60 γ	120 γ	240 γ	Mean	
	(mg/100 ml.)					
Sodium						
G[b]	335	333	337	334		4
H[b]	339[c]	338	343	338	337	4
Potassium						
G	12.0	12.5	12.3	12.7		0.3
H	12.4[c]	12.5	12.8	12.4	12.4	0.3
Inorganic phosphorus						
G	1.66	1.79	1.66	1.75		0.16
H	1.83[c]	1.92	1.66	1.65	1.74	0.16
Calcium						
G	4.9	5.1	5.1	4.9		0.4
H	5.1[c]	5.2	5.0	5.1	5.1	0.4

[a] γ/lb live weight/day.
[b] G, Guernseys; H, Holsteins. The value under each intake represents the mean concentration obtained from the cerebrospinal fluid of three calves.
[c] Cerebrospinal fluid sample not obtained from one calf, missing value calculated (3).

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EFFECT OF DURATION OF EXPERIMENT ON EXPERIMENTAL ERRORS IN CALF NUTRITION GROWTH STUDIES[1]

Estimates of experimental errors of growth are of importance for efficient planning and adequate interpretation of calf nutrition studies. For example, they are of value in estimating the number of calves required per treatment to obtain a specified statistically significant difference in growth between treatments (2, 4). As early as 1913, Mitchell and Grindley (5) demonstrated that the size of the experimental errors for increases in live weight, expressed as percentages of mean gains, of beef cattle, sheep, poultry, and swine, decreased with increasing time on experiment. The rates of change of these decreases were most rapid during the early parts of the experiments and, thereafter, were at considerably slower rates or had a tendency to approach constant values. Somewhat similar findings have been reported (3) for increases in several linear growth measurements as well as live weight of dairy calves fed a single ration, uniformity data, and for increases in live weight of dairy heifers (1). The present study was undertaken to determine the effect of duration of experiment on the experimental errors of increases in growth of dairy calves to which various dietary treatments had been imposed, in contrast to the aforementioned estimates obtained from uniformity data (3).

Data used in this study were from seven randomized block and one nonreplicated factorial experiment conducted at the Storrs Station from 1949 through 1956. As they became available, one-day-old calves from Connecticut State institution and private herds were assigned to treatment according to previous random allotment. In all but one experiment (a randomized block), breed and sex were included as treatment, so that the existence of possible interaction between these and other treatment effects might be ascertained. The number of calves per experiment ranged from 15 to 36. The number of degrees of freedom for the error mean squares ranged from four to 21.

All calves were maintained in individual tiestalls throughout the course of their respective experimental periods. After nursing their dam or receiving colostrum from a frozen colostrum bank, calves in all but one experiment were fed a limited amount of whole milk, 168 to 182 lb., to the 35th day of age, a limited amount of starter, 2.5 to 4.0 lb. per calf per day, and ad libitum amounts of hay or ad libitum amounts of various mixtures of starter and hay. In the one experiment, one-half of the calves received milk as above, and one-half received milk at a daily rate of 10% of live weight up to a daily maximum of 10 lb. per calf per day to the 63rd day of age. The nutritional treatments include

[1] This study was supported in part with funds provided by Wirthmore Feeds Inc., Waltham, Mass. The authors are grateful to Mrs. Mae Miller, Mrs. Elaine Trantum, R. T. Chatterton, Jr., A. P. Grifo, Jr., H. A. Kemmerer, Jr., and R. Teichman, for assistance in computations. We are especially indebted to Dr. R. L. Anderson for discussing the statistical aspects of this study.

type and/or level of roughage in four experiments, chlortetracycline levels in starter in two trials, and level of tallow in starter and milk allowance and type of roughage, each in one study.

Live weights were observed to the nearest 1 lb. at birth and on the last day of each successive seven-day age period. Height at withers, heart girth, and girth of paunch were measured to the nearest ¼ in., as previously described (3), on the seventh day of age and on the last day of each successive 14-day age period for five of the eight experiments. The statistical methods used were those of Snedecor (6) and Cochran and Cox (2). The statistics reported herein are for growths between seven and 91 days of age for seven of the experiments and between birth and 84 days of age for the eighth experiment. The use of the data from the eighth experiment appeared to be justified, since a previous study (3) indicated that the decrease in the magnitude of the coefficients of variation of growth with time on experiment was not associated with age of the calf.

In Table 1 are presented the medians and ranges for the mean growths, the standard deviations, and coefficients of variation. As would be expected, the magnitude of the growths increased markedly with increases in duration of experiment. Although the standard deviations also increased with increases in the length of the experimental period, these increases were not as rapid as for growths. Thus, the coefficients of variation decreased with duration of experiment. These decreases were most marked during the early portion of the experimental period. After the 56th day or 8th wk. on experiment, the decreases for the medians became more or less constant in magnitude, decreasing between one and six percentage units per 14-day increase in duration of the experiment. As would be expected with the limited number of experiments, the upper and lower limits of the ranges exhibited greater variation in these decreases which amounted to between +3 to −9 percentage units.

The trends in the decrease of the coefficients of variation of growths are in agreement with those for calves fed a single ration (3), as well as those for other classes of livestock (5). Since experimental periods of greater than 84 days' duration might have resulted in further decreases in the magnitude of the coefficients of variation, additional calculations were made

TABLE 1

Median and range of mean growth ($\bar{y}$), standard deviations for growth (S.D.), and coefficients of variation (C.V.) as affected by duration of experiment

Growth criterion		Days on experiment					
		14	28	42	56	70	84
Live weight *(lb.)*							
$\bar{y}$	Median	9.3	23.8	40.8	63.4	87.2	107.6
	Range	2.1–12.5	11.8–32.5	26.7–48.9	46.4–72.0	69.0–97.7	89.4–127.9
S.D.	Median	5.0	7.3	10.4	12.4	13.0	14.0
	Range	3.4–7.1	4.7–12.6	7.6–13.0	9.4–16.4	11.1–20.2	12.8–23.4
C.V.	Median	58	34	24	21	17	14
	Range	34–219	21–56	18–42	13–27	11–23	10–20
Height at withers *(in.)*							
$\bar{y}$	Median	0.73	1.45	1.99	2.84	3.88	4.81
	Range	0.47–0.87	1.30–1.62	1.69–2.52	2.64–3.36	3.30–4.46	4.47–5.28
S.D.	Median	0.39	0.53	0.75	0.81	0.82	0.86
	Range	0.27–0.75	0.20–0.82	0.23–0.89	0.65–0.90	0.65–0.94	0.75–0.98
C.V.	Median	53	38	39	28	23	19
	Range	31–160	12–63	9–53	23–33	16–28	14–22
Heart girth *(in.)*							
$\bar{y}$	Median	0.92	2.23	3.53	4.84	6.43	7.86
	Range	0.45–1.73	1.61–2.86	2.89–4.51	4.08–6.08	5.86–7.74	7.42–9.17
S.D.	Median	0.96	1.11	1.08	1.25	1.42	1.37
	Range	0.84–2.25	0.44–1.78	0.84–1.31	1.17–1.89	1.14–1.61	0.94–1.53
C.V.	Median	130	63	30	27	21	17
	Range	73–198	15–84	21–43	25–33	19–24	11–18
Girth of paunch *(in.)*							
$\bar{y}$	Median	2.00	5.90	10.49	13.19	16.06	18.04
	Range	1.39–3.85	5.17–7.74	9.69–11.98	12.14–14.61	14.94–17.41	17.80–19.75
S.D.	Median	1.52	2.02	2.22	2.00	2.33	2.39
	Range	1.24–1.88	1.49–2.77	1.80–2.65	1.82–2.73	1.93–3.60	1.91–2.86
C.V.	Median	72	29	20	16	15	13
	Range	48–98	19–54	15–27	14–19	13–22	11–16

where possible. In five of the eight experiments in which the duration of experiment was greater than 84 days, the median coefficients of variation of live weight increases for an 84- and a 98-day period were, respectively, 14 and 14, with a range for both values between 10 and 15. Although these values indicated that little further decrease would be expected, additional data consisting of longer experimental periods are needed to verify these results.

The possible effect of large differences in growth between treatments on the magnitude of the experimental errors was explored by plotting the coefficient of variation against the treatment difference, divided by the experiment mean times 100. Neither within a particular duration of experiment group nor across all durations was there a noticeable trend. Although admittedly, the number of experiments, eight for live weight gain and five for linear growth, was few, these findings do not contest the contention of Mitchell and Grindley (5) that the severity of treatment apparently does not affect the size of the experimental errors of growth.

To detect a specified statistically significant difference in growth, fewer calves are needed per treatment as the duration of the experiment is increased. To illustrate this, the median coefficients of variation, as well as the lower and upper range of values from Table 1, were used to calculate (2) the number of replications required to detect a growth difference of 20% of mean growth for experimental periods ranging from 42 to 84 days' duration (Table 2). As can be seen from these estimates, the number of replications when using the median coefficients of variation decreased by approximately two-fifths for live weight, one-fourth for height at withers, one-third for heart girth, and one-half for girth of paunch, when the experimental period was increased from 42 to 84 days. When using the upper range values of the coefficient of variations, the magnitude of the decreases in the number of replications required was considerably greater; whereas, when using the lower range values, the decreases were less and also more variable.

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TABLE 2

Effect of duration of experiment upon the number of replications required to detect a significant difference in growth of 20% of the mean growth[a]

Growth criterion	Days on experiment			
	42	56	70	84
	(No. of replications)			
Live weight				
Median	25	20	14	10
Range	15–72	9–31	7–23	7–18
Height at withers				
Median	62	33	23	17
Range	6–113	23–45	13–33	10–21
Heart girth				
Median	38	31	20	14
Range	20–75	27–45	17–25	7–15
Girth of paunch				
Median	18	13	11	9
Range	11–31	10–17	9–21	7–13

[a] Using the median and range of the coefficients of variation from Table 1, and assuming randomized block design with two treatments, two-tailed "t" test, 5% significance level, and 80% probability of detecting difference (2).

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OUR INDUSTRY TODAY

CURRENT DIETARY RECOMMENDATIONS FOR THE PREVENTION AND TREATMENT OF CORONARY HEART DISEASE AND WHAT THEY MEAN TO THE DAIRY INDUSTRY[1]

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Dietary recommendations for treatment of coronary heart disease are encountered almost daily in professional journals, medical books, dietary manuals, books for the lay public, popular magazines, and newspapers. The recommendations are made by researchers working in the field, researchers evaluating the work of others, organizations such as the American Medical Association and the American Heart Association, public health nutritionists, public health physicians, physicians in private practice, and the employees of some food and drug companies in the promotion of certain products.

The most general recommendation with which all seem to agree is to avoid excess body weight by controlling food intake and exercising regularly. Frequently coupled with this is a recommendation to restrict fat intake as a means of reducing total calorie intake. Some researchers, physicians, public health nutritionists, and commercial companies carry this further. They advise people to avoid animal fats, as in the visible fat of meats, eggs, lard, butter, ice cream, cheese (except dry curd cottage cheese), and whole milk; to avoid hydrogenated shortenings, oleomargarine, coconut oil and cocoa butter; to use nonfat milk solids, egg whites, lean meat only, poultry, and fish as sources of protein; to use vegetable oils such as corn, cottonseed, peanut and safflower seed oils, and some new vegetable oil products made as a substitute table spread to replace butter and oleomargarine. These recommendations are made to the population as a whole by some investigators. Others limit the recommendations to individuals who have high serum cholesterol levels, high atherogenic index, a family history of coronary heart deaths, clinical symptoms of atherosclerosis, or have had a coronary heart attack, are obese, have diabetes, are hypothyroid, smoke, have high blood pressure, or work and live under stressful circumstances.

These recommendations are based on evidence which suggests that high serum cholesterol levels may, in some way, increase the susceptibility of the individual to atherosclerosis and to coronary thrombosis and occlusion; and on changes in serum cholesterol levels which are related to changes in kind and amount of dietary fat. The major problem is that all information does not point in the same direction. Much evidence is conflicting. Many very competent researchers and physicians do not agree with these broad recommendations for drastic changes in the American diet. Indeed, the Food and Nutrition Board of the National Research Council, National Academy of Sciences, in its Recommended Dietary Allowances, Revised 1958, published this statement:

"Much attention has been given to the potential harmful effects of excessive intakes of fat, or of some kinds of fat. In the United States, with its relatively high mortality rate from coronary heart disease, and with a large proportion of food calories derived from fat, much interest has attached to findings on the subject, and broad proposals have been made for correcting the national diet. The present knowledge of fat metabolism and of the relation of dietary fat to the occurrence of disease has been recently reviewed by the Committee on Fats in Human Nutrition of the Food and Nutrition Board of the National Research Council. It is not yet possible to state definitely a reasonable allowance for fat in the diet or to indicate the characteristics of a fatty acid mixture most favorable for the support of health. A diet selected from a wide variety of foodstuffs, both vegetable and animal, is most like to maintain good health." (19)

In another bulletin published by the Food and Nutrition Board under the title "The Role of Dietary Fat in Human Health," the following statement appears:

"Until it is clear which fats are more desirable nutritionally and which, if any, are undesirable—major changes in American dietary habits are not to be recommended.

"There is nothing in the history of nutrition that should persuade man to give up reasonableness and moderation in his choice of food. Above all, there is need of judicious and accurate interpretation of dietary observations. A wider choice of those foodstuffs shown by experience and by experiment to be useful components of the daily ration is the best possible protection against nutritional disease." (20)

These statements of the Food and Nutrition Board of the National Research Council are not

[1] Reprints available for 90 days after publication of this paper. For prices, see page 1434 of this issue.

[2] Presented at Dairy Technology Conference, University of Illinois, Urbana, March 26, 1959.

particularly palatable to some individuals with whose opinions they conflict. The conflict has been dealt with in a very interesting way in a book from which the following excerpt was taken:

"The platitude issued by some supposed experts in nutrition on the danger of tampering with the existing American diet should not be taken at all seriously, since it is entirely possible to plan a diet that is highly adequate. Indeed such diets can be more than adequate by National Research Council Standards, which reflect the best available nutrition knowledge." (7)

Here, the membership of the Food and Nutrition Board of the National Research Council is referred to, in the first sentence as "some supposed experts in nutrition"; and in the second sentence as reflecting "the best available nutrition knowledge." Unfortunately, the reader for whom the text was prepared will not recognize these and other discrepancies.

DAIRY INDUSTRY DILEMMA

All recommendations pertaining to diet in relation to heart diseases have implications for the dairy industry, as they affect consumer attitudes toward dairy foods and as they present opportunities to the dairy industry to tailor-make products which might further benefit mankind and sustain a stable economy for the industry. Certainly, of all animal food industries, the dairy industry is in the best position to provide nutritious and palatable foods tailor-made to meet whatever needs might be established. Milk is the only animal food from which part or all of the fat can readily be removed, modified, or replaced. However, the dairy industry is so vital to human health and to our agricultural economy, that broad changes in basic dairy foods must rest firmly on positive and conclusive evidence . . . and ought not to be made in absence of conclusive evidence of need.

If evidence in this area was conclusive in any direction, and if there was general professional agreement on dietary recommendations, the implications would be clear-cut and the dairy industry could leave its products alone . . . or modify them to meet medical and consumer demands. As it is, evidence is conflicting. There is no rational basis at the present time on which the dairy industry might make changes, other than those which it has already made to meet demands by providing: low-fat milks fortified with vitamins A and D and nonfat milk solids, low-fat frozen desserts, low-sodium milks, dry-curd cottage cheese, and nonfat milk solids which can be incorporated into many different products. Other experimental products have been and are being studied but are not generally available on the market.

Dr. Harold Hansen Mitchell, eminent nutritionist for half a century, has said, "A hypothesis is good only as long as there is no evidence against it." How conflicting is evidence pertaining to the hypothesis that coronary heart disease is caused by a high intake of animal fat?

First, while data have been cited which show a correlation between fat consumption in selected countries and incidence of coronary heart disease . . . when data from other countries are considered, this correlation no longer exists. This is clearly shown in Table 1, which presents data on National Food Supplies and Deaths from Heart Disease from ten different countries. The data in this table do not reveal any relationship between national food supplies and death rates from arteriosclerotic and coronary heart disease.

It is of interest that one investigator who tells the American public that "no country which has a high incidence of coronary heart disease has a low intake of dairy foods" has also said "great differences between countries in mortality from arteriosclerotic and coronary heart disease are not quantitatively related to differences in per capita milk and dairy foods consumption." Although he places greater importance on the former statement, the latter one is far more significant. No one has died of coronary heart disease without first being born . . . yet stating that being born is the cause of dying does not aid materially in placing the immediate cause of death of anyone.

Secondly, recommendations regarding dietary fat are aimed at attempts to lower serum cholesterol levels or to modify the "atherogenic index" of individuals, on the assumption that development of atherosclerosis can be prevented or reversed by so doing. This is based on evidence of high serum cholesterol levels and abnormal "atherogenic index" being associated in population groups with a high incidence of coronary heart disease and being associated with diseases like diabetes, in which coronary heart disease is also frequently observed. Yet, it is generally agreed that serum cholesterol values can not be used to predict accurately the presence or severity of atherosclerosis, or who will or will not have a coronary heart attack. Many young people with low serum cholesterol levels die of a heart attack. Many others with high serum cholesterol levels live to an old age. Two reports in the literature show no correlation between serum cholesterol values and severity of atherosclerosis as evaluated at autopsy (14, 16). Only one laboratory claims predictive value for the "atherogenic index," which is considered by others of even less value than serum cholesterol levels (6, 7). The "atherogenic index" is fur-

TABLE 1

National food supplies and deaths from heart disease

Country	Sex	Deaths from heart disease (including arteriosclerotic and coronary heart disease) per 100,000 population of each sex in 1951[a]			Per capita consumption whole milk equivalents per cent milk fat basis		Available per person per day in 1952, 53, or 54 from national average food supplies[c]								
					3.87% milk fat[b]	Country standard[b]	Calories	Total protein	Animal protein	Total fat	Animal fat	Vegetable fat	% calories total fat[d]	% calories animal fat	% calories vegetable fat
		(All ages)	*(35–39 years)*	*(50–54 years)*				(g)					(%)		
United States	M	342.0	50.3	452.8	699	699	3090	91	63	135	95	40	39	28	11
	F	203.5	14.1	127.5											
Finland	M	230.5	46.8	426.3	1470	1423	3100	96	51	103	86	17	30	25	5
	F	153.3	18.6	111.5											
Canada	M	276.8	38.4	365.1	937	1027	3110	95	59	130	101	29	40	29	11
	F	167.2	12.3	90.4											
Australia	M	302.0	24.1	308.7	1090	1096	3290	95	63	122	106	16	33	29	4
	F	201.1	9.0	94.2											
Italy	M	148.2	22.5	144.2	283	297	2600	80	22	60	29	31	31	10	11
	F	161.0	24.1	82.7											
Denmark	M	204.6	7.1	139.9	888	857	3240	91	50	141	104	37	39	29	10
	F	174.7	3.8	44.3											
Sweden	M	212.4	9.3	136.5	1150	1173	2980	86	55	128	110	18	39	33	6
	F	193.2	4.4	53.6											
Norway	M	128.0	11.9	123.6	927	934	3120	90	50	131	99	32	38	29	9
	F	107.8	4.0	29.9											
Netherlands	M	145.6	7.4	120.1	565	616	2860	81	42	116	52	64	37	16	21
	F	127.6	2.0	36.5											
Japan	M	40.9	23.8	81.6	18	24	2010	58	13	18	5	13	8	2	6
	F	42.6	30.7	67.9											

[a] From official vital statistics assembled by Statistical Division of World Health Organization, Geneva, Switzerland. Includes deaths listed under International Code No. 420, 421, 422. No. 421 reflects heart disease of different origin than arteriosclerotic and coronary heart disease.

[b] Foreign Agricultural Circular, October 17, 1955, and personal communication. Foreign Agricultural Service. U. S. Department of Agriculture.

[c] Data assembled by Nutrition Division of Food and Agriculture Organization, Rome, Italy. Food consumed in 1952, 53, or 54 could not influence death rates in 1951. However, these data do reflect food patterns of previous years for which comparable data are not available. Data are used here only to compare national food patterns and not as absolute values relevant to cited mortality.

[d] Other sources indicate the following per cent of calories from total fat: United States, 40%; Canada, 37.5%; Australia, 38%; Italy, 20%; Sweden, 37%; Netherlands, 32.5%.

ther complicated by its author's claims that it might indicate either a need for a low-animal-fat or a low-carbohydrate diet. He claims that vegetable oils have no effect on "atherogenic index" and, therefore, can be used to replace both animal fats and carbohydrates (7).

Third, by assuming that it may be desirable to reduce serum cholesterol levels by modifying the kind of fat consumed in the diet, then the contradictions increase. In human subjects, the constituents of dietary fats have been shown to affect serum cholesterol levels as follows:

saturated fatty acids both lower . . . and elevate cholesterol levels (11);

oleic acid, the mono-unsaturated fatty acid, has no effect . . . but with animals it elevates cholesterol levels (8, 11);

the more highly unsaturated a fat as judged by iodine number, the more it depresses cholesterol levels (1);

total unsaturation, as judged by iodine number, is not important . . . it is the essential fatty acid, linoleic acid, which depresses cholesterol levels (13);

hydrogenating corn oil . . . though it reduces total unsaturation and linoleic acid content and increases saturated fatty acid content . . . does not rob the oil of its ability to lower cholesterol levels (2);

adding a highly saturated fat to a highly unsaturated oil does not rob it of its ability to lower cholesterol levels (11).

Milk fat tends to elevate blood cholesterol levels. This is not due to its cholesterol content . . . there is general agreement that dietary cholesterol does not influence blood cholesterol levels in human beings.

Could this effect of milk fat be due to its relatively low level of unsaturation as judged by iodine number? This is claimed by many . . . yet normal healthy babies fed cow's milk containing fat with an iodine number around 38 have no higher blood cholesterol values than normal healthy babies fed human milk containing fat with an iodine number of 62 . . . and no higher blood cholesterol values than babies recovering from severe protein deficiency disease while consuming nonfat milk solids and no fat at all . . . about 175 mg. cholesterol per 100 ml. blood serum. Meanwhile, normal healthy babies fed nonfat milk solids combined with vegetable oils have very low blood cholesterol levels . . . levels similar to those of babies with severe protein deficiency who are not responding to dietary treatment . . . about 110 mg. cholesterol per 100 ml. of blood serum (3, 21).

Could it be due to the oleic acid content?

One investigator says "No" . . . that blood cholesterol levels in adult men on controlled diets are not affected by dietary oleic acid (12). Others have assumed oleic acid has no effect. Yet, oleic acid content of human and cow's milks are very similar, 36% and 32%, respectively . . . and babies fed these two milks have comparable cholesterol levels (3, 15). Also, animals fed oleic acid tend to have higher cholesterol levels than when fed either saturated or polyunsaturated fatty acids . . . and animals deficient in essential fatty acids tend to accumulate the metabolites of oleic acid in their heart tissue even though on a fat-free diet (9, 11).

But from this contradictory evidence . . . do we have any basis for proposing any replacement for milk fat or any modification of milk fat itself? Some researchers and physicians are saying "Yes." The Food and Nutrition Board of the National Research Council says "No." I should like to ask each person to examine the evidence and form his own conclusions. Certainly, I feel unprepared to suggest any "improvement" on milk fat at the present time.

Fourthly, many factors of diet and physiology also influence blood cholesterol levels, including:

kind and amount of dietary protein;
kind and amount of carbohydrate;
amount of magnesium and ratio of magnesium to calcium;
various vitamins including B_6, C, D, and E; and
hormonal balance as affected by insulin, thyroid function, and sex hormone secretions (11, 25).

Time does not permit going into the details of how each of these factors influences blood cholesterol. Suffice it to say that:

diabetics examined at autopsy showed a 30% incidence of myocardial infarction . . . heart damage from a coronary occlusion . . . as against a 5% incidence in the nondiabetic autopsy population (24);

hypothyroid function produced in baboons was more effective in producing atherosclerosis in that species than any other measure studied . . . dietary or physiological (5);

the greatest differences observed in mortality rates in the United States are differences between sexes (see Table 1).

Is it conceivable that nature has combined a high quality protein with a good source of magnesium and calcium and other essential nutrients with a fat which is harmful? It is unreasonable to believe so and evidence has not established that it is so. Certainly, in the face of conflicting evidence the dairy industry can have faith in all constituents of its product, milk.

Evidence does not favor milk fat or dairy foods being in any way responsible for our

high incidence of coronary heart disease. The United States, as noted in Table 1, is first among countries in mortality rate from heart disease . . . but ranks about 13th in per capita consumption of whole milk equivalents from all dairy foods, as shown in Table 2 (10).

TABLE 2

Whole milk equivalent consumed per person, 1956

Country	*Pounds*
1. Ireland	1,534
2. New Zealand	1,428
3. Finland	1,336
4. Australia	1,023
5. Sweden	1,013
6. Canada	1,012
7. Switzerland	980
8. Belgium	975
9. Norway	899
10. Denmark	842
11. United Kingdom	764
12. France	721
13. UNITED STATES	707
14. West Germany	704
15. Netherlands	668

The cry today is against a rising incidence of arteriosclerotic and coronary heart disease, but what of the changing picture in dairy foods consumption in the United States?

Figure 1 shows the relationship of pounds of milk fat to pounds of solids-not-fat and pounds of total milk solids consumed per person in the United States since 1925. Starting about 1935, there was a slow increase in per capita consumption of nonfat milk solids and total milk solids to about 1945, with a small decline about 1948, with little change in the past 10 yr. Per capita consumption of milk fat remained almost constant through 1945, but has shown a small but steady decrease since then (10).

Table 3 shows that, today, per capita consumption of milk fat is about 1.1 oz. of milk fat per person per day, equal to about 2.5 tablespoons of butter per person per day. This is 5 lb. per person per year less than it was from 1925 to 1946. This milk fat has been replaced by about 8 lb. or more of vegetable oils in the form of hydrogenated margarines and shortenings and salad and cooking oils (10, 22, 23).

How much influence have dietary recommendations to avoid milk fat had to do with this change of form of fat consumed? Who knows? One could assume very little if one considers that the major recommendation has been to reduce total fat intake . . . and the total fat purchased for family consumption increased from 38% of the calories in 1936 to 44% in 1955 (4).

One might expect the pattern of dairy foods consumption to have changed radically toward greater consumption of low-fat products. Certainly, many of our dairy companies have

TABLE 3

Milk fat, solids-not-fat, and total milk solids
Consumption per person, United States

Year	Pounds		
	Milk fat	Solids-not-fat	Total milk solids
1925–29	31.7	37.8	69.5
1935–39	31.7	39.6	71.3
1945	31.6	48.7	80.3
1950	29.4	45.9	75.3
1951	28.2	45.9	74.1
1952	27.3	47.0	74.3
1953	26.8	46.4	73.2
1954	27.1	47.5	74.6
1955	27.3	47.9	75.2
1956	27.1	48.5	75.6
1957	26.5	48.3	74.8

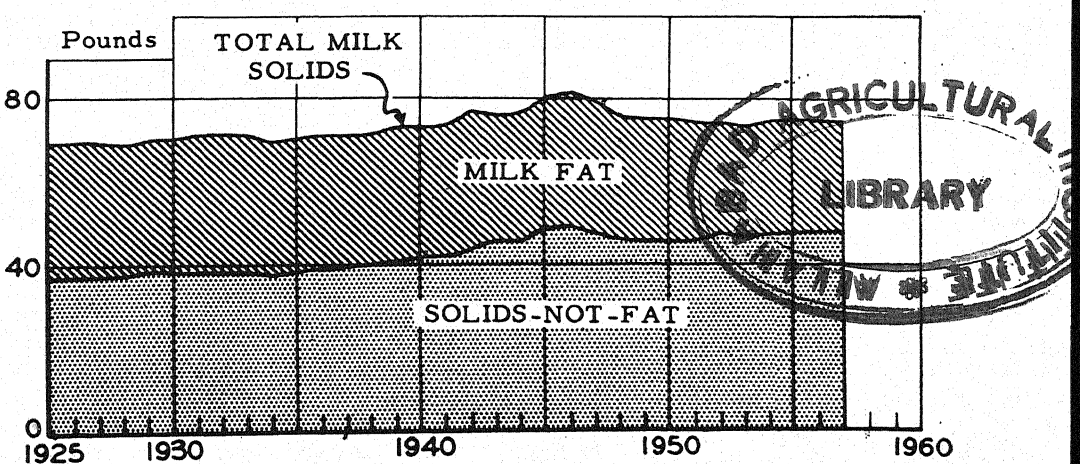


FIG. 1. Pounds of milk fat, solids-not-fat, and total solids consumed per person in the United States since 1925.

watched sales of low-fat milks increase. But what of the country as a whole?

Table 4 shows that since 1929, consumption of fluid milk has increased from 126 to 142 qt.

TABLE 4

Consumption per person, United States

Year	Milk	Nonfat milk	Buttermilk	Chocolate milk	Cottage cheese
	(qt.)				
1925–29	126	8.1	23.0	....	1.1
1935–39	123	7.7	21.9	1.2	1.5
1940	123	7.4	20.7	....	1.9
1945	156	6.4	19.1	....	3.0
1946	150	6.3	18.5	....	2.9
1947	142	6.2	18.7	....	2.6
1948	137	5.9	17.9	....	2.9
1949	138	5.7	17.6	3.4	3.2
1950	136	5.5	17.2	3.7	3.5
1951	139	5.2	16.5	3.8	3.9
1952	141	5.3	16.6	3.9	4.0
1953	140	5.3	16.5	3.9	4.2
1954	140	5.3	16.5	3.9	4.4
1955	142	5.4	16.4	4.3	4.5
1956	143	5.4	16.3	4.5	5.1
1957	142	5.4	16.4	4.1	5.2

per person. There was an all-time high in 1945 of 156 qt. per person, which declined by 1947 to 142 qt., approximately today's level of consumption (10).

Consumption of fluid nonfat milk actually declined since 1925. The 1925 to 1929 year average was about 8 qt. per person. This declined by 1950 to a level of 5.5 qt. per person per year. National statistics indicate that per capita consumption of fluid nonfat milk has remained fairly stable since 1950. We have a similar picture for buttermilk, except that the consumption of buttermilk is about three times as great as the consumption of fluid nonfat milk. Consumption of chocolate milk has remained somewhat stable since 1950, too. Consumption of cottage cheese has shown a definite and regular trend toward increased consumption (10).

This table does not reflect the increased availability and consumption of the partially skimmed fluid low-fat milks. At the present time we have products on the market which vary from both whole and skimmed milks in butterfat and nonfat milk solids content. With increasing interest in the fat intake of the diet, it may be desirable to mark bottle caps or carton containers with the actual level of nonfat milk solids and butterfat content of modified fluid milks. It is conceivable that we may, in the future, have an increased consumption of many of the specialty dairy foods. In the last few years, in answer to consumer demand created by medical recommendations, dry curd cottage cheese is being marketed through consumer outlets. While it may have been available in some markets in the past, it is only now that it has been generally available.

Table 5 shows what has happened to consumption of fluid cream. In the United States, per capita consumption of cream has dropped to about a third the level that it was before World War II. Consumption has leveled off at about 47 lb. of fluid milk equivalent consumed as fluid cream per year for the last 5 yr. (10).

The story is about the same with butter. The gratest decline in consumption of milk fat has been through decreased use of fluid cream and butter. At the present time, we are consuming less than half of the amount of butter people consumed 30 yr. ago. Consumption has leveled off between 8.5 and 9 lb. of butter per person per year in the United States (10). In view of all of the furor of an attempt to blame an increasingly high rate of heart disease in the United States on consumption of butter, these figures do not tally very well.

The trends in consumption of cheese are toward a small but steady increase, with a leveling off of consumption at about 8 lb. of cheese per person per year (10).

Consumption of ice cream only has increased considerably over that of 20 yr. ago. However, consumption has been pretty well leveled off since 1950, showing only small increases in consumption of ice cream per person per year. We have had an increase in consumption of related products. These have increased from about 3 to about 5 qt. per person per year. These products would include the low milk fat products, such as ice milks and milk sherbets (10).

The inclusion of modified vegetable oils in imitation of ice milks and ice creams is worth noting here. The fat which has largely been used to replace milk fat in ice cream has been a partially hydrogenated coconut oil. These products have no recognized advantage over ice cream, if consideration is to be given with respect to the dietary recommendations reviewed here earlier.

While dry whole milk consumption has increased fourfold since 1925, the consumption of nonfat dry milk, in pounds, is more significant. This has increased eightfold, leveling off in the last 3 yr. at about 5.5 lb. per person per year. Meanwhile, there has been a small but steady decline in the consumption of evaporated milk, with condensed milk holding fairly steady over the past 10 yr. or more (10).

THE FUTURE

We have reviewed what has happened during the past 25 yr. with respect to dairy foods consumption. Many factors have influenced these changes: nutrition education, economic, technological and social progress. We can anticipate that many more changes will occur in the future.

The crusading efforts to induce people to avoid milk fat in various dairy foods have been intensified in recent months. This is creating

TABLE 5
Consumption per person, United States

Year	Cream	Butter	Ice cream		Cheese	Nonfat dry milk
			Ice cream only	Ice cream and related products		
	(lb.) (milk equiv.)	*(lb.)*	*(qt.)*	*(qt.)*	*(lb.)*	*(lb.)*
1925–29	68	18.0			4.6	.7
1935–39	66	17.0	8.4	8.7	5.6	1.9
1940	66	17.0	9.7	10.3	6.0	2.2
1945	64	10.9	13.4	16.9	6.7	1.9
1946	66	10.5			6.7	3.3
1947	63	11.2			6.9	2.9
1948	60	10.0			6.9	3.3
1949	56	10.5			7.3	3.3
1950	56	10.7	14.6	16.8	7.7	3.7
1951	53	9.6	14.8	17.4	7.2	4.2
1952	49	8.6	14.9	18.5	7.6	4.6
1953	47	8.5	15.0	19.2	7.5	4.1
1954	47	8.9	14.5	19.2	7.9	5.1
1955	47	9.0	15.0	20.0	7.9	5.5
1956	47	8.7	15.0	20.2	8.0	5.5
1957	46	8.4	14.9	19.8	7.8	5.5

a demand for low-fat products. It could seriously undermine the market for standard dairy foods. The extent to which it does will depend upon the interest and credulity of the public, and the extent to which they are exposed to counter recommendations. Even though they are much improved today, low-fat and fat-free products are not as palatable as those which contain milk fat. Therefore, any large consumer shift to such products may tend to reduce dairy foods consumption as well as milk fat consumption, unless the products can be made highly palatable and the consumer be given assistance on how to use them to the best culinary advantage.

The dairy industry is dependent upon the production of a natural foodstuff by a highly specialized farm animal. Through breeding and feeding techniques, it is possible to modify the quality and quantity of the product which the cow produces, within limitations. However, these changes do not always go just exactly the way some individuals might decide they would like to see them go. For instance, by breeding a dairy herd for high milk fat content, we simultaneously breed them for a high nonfat milk solids content. By breeding them for a low milk fat content, we simultaneously breed them for a low nonfat milk solids content. It may be possible, by means of very careful selection, to breed for a better ratio of nonfat solids to milk fat. To what extent this can be done is not known at present. We do have evidence from some parts of the United States at the present time that our long-time trend toward breeding for volume production with a lower butterfat content is actually leading to a lower nonfat milk solids concentration. This has become a problem in some areas where, for the first time, some milks are produced which are not meeting the legal minimum standard for concentration of nonfat milk solids (18).

Regardless of the ultimate aims of our breeding and feeding, changes in the dairy industry do not come quickly . . . nor can they . . . nor should they. No food industry has been more carefully controlled as to the identification of its products than has the dairy industry. While there are many people who would like to explain this careful control as some kind of a fetish or monopoly of the dairy industry, actually the legislative control of milk production, handling, and marketing is for the consumer's protection. Changes which are made with regard to the concentration of nutrients or the modification of nutrients in dairy foods . . . or changes which might be made in legislative control over the products or their identification must be based on very sound ideas. If the dairy industry can serve the American public, its consumers, better by modification of products, then I am certain that the dairy industry should and will meet consumer demands. However, they should have a very good reason for making any changes that are effected. At the present time, we do not have a sound basis for further modifying our dairy foods with respect to their milk fat content.

Just as we have no basis for modifying our dairy foods beyond the modifications that have already been made, we do not have any basis for changing our dietary recommendations. You have seen from the foregoing that there is no general agreement and no basis for general agreement regarding the desirable kinds and amounts of fat in our diet. This is the evaluation of the Food and Nutrition Board of the National Research Council. It is also the evalu-

ation of the American Heart Association, and the Council on Foods and Nutrition of the American Medical Association.

The dairy industry and its representative Research, Education, and Public Relations agency, the National Dairy Council and its affiliated Dairy Council units, must be guided by group professional thought rather than individual professional thought. Therefore, we are guided by the recommendation of the Food and Nutrition Board of the National Research Council, the Council on Foods and Nutrition of the American Medical Association, the Agriculture Research Service of the U. S. Department of Agriculture, the United States Public Health Service, the Federal Food and Drug Administration, and other authoritative bodies, with respect to the kinds of dietary recommendations which we make to the American public directly and through professional people.

Reproduced here (Figure 2) is National Dairy Council's new "Guide To Good Eating." This is patterned generally after a daily food plan of the U. S. Department of Agriculture. It bears a statement of review and acceptance by the Council on Foods and Nutrition of the American Medical Association.

At the present time, there is nothing in any of the materials from which we have quoted today which detracts one iota from the validity of the recommendations on the National Dairy Council's "A Guide To Good Eating." Like the Food and Nutrition Board of the National Research Council, this piece of material and all other materials developed by National Dairy Council are subject to revision as new knowledge accrues, permitting further progress which will permit and encourage the development of good food practices by the population of the United States and will allow for greatest dividends in health and in disease prevention.

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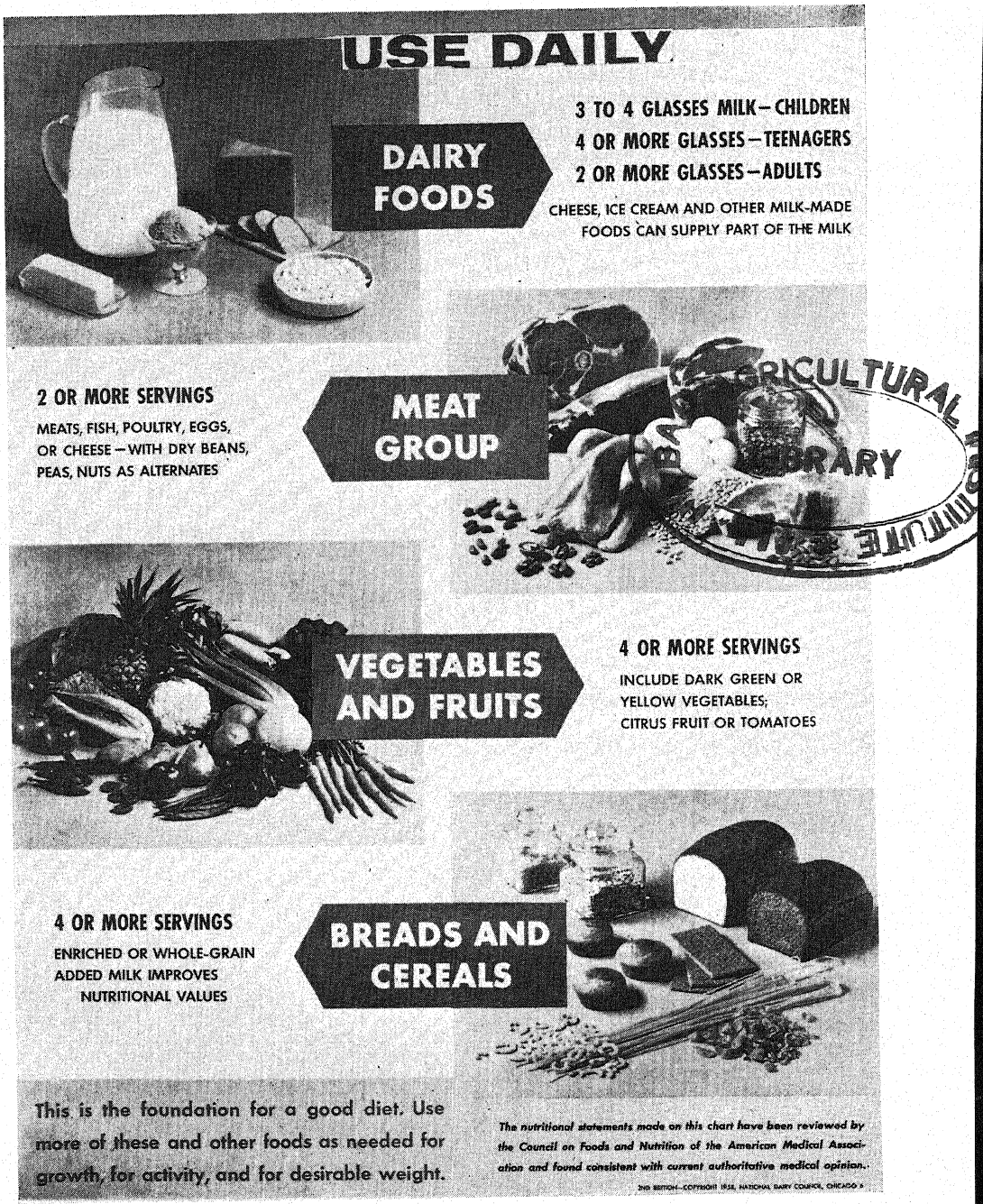


FIG. 2. A guide to good eating.

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NEW IDEAS IN EVAPORATORS AND DRYERS

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This material on new ideas will be divided into three major categories. First, the necessary foundation for present or future development. Secondly, the five major subjects involved in development. And thirdly, some specific items of major subjects for evaporators and dryers.

The foundation of development, particularly in the field of evaporation and drying, is the ability to predetermine and control certain conditions within the machines. If these conditions vary for any reason it is, in effect, changing the entire design of the equipment. These conditions are pressure within the first effect and the liquid level control in the evaporator.

In a dryer, these conditions are a given delivery of air in terms of pounds, a given heat release resulting in a precise air temperature, a specific static pressure within a drying chamber, continuous product removal, and continuous operation of dust removal equipment. These conditions demand the use of instrumentation and automation to create an index from which to work.

One of the greatest hindrances, from an engineering standpoint, is the reluctance to accept instrumentation and automation. This reluctance is in many cases well founded, due to preliminary efforts which have failed and which have caused damage to product and equipment. It is noteworthy to investigate the two most prominent reasons for failure. First, most automatic equipment depends either upon compressed air or upon electric current to actuate the controls. This we must consider the source of power, which will be either a plant air supply or primary electrical system or, possibly, both. The greatest source of failure in air-operated instruments is water and oil in the air supply system, and the greatest source of failure in electrically operated instruments is unstable voltage. In a vast majority of cases, either of these conditions can be corrected without major expenditure and should be on a preventive maintenance basis.

When we consider other industries, such as metal spray plants, we realize that a minute amount of contamination in the air supply would virtually put this group out of business. This places the instrument problem as a basic plant engine-room fault, rather than an individual control problem.

Electrically operated instruments follow the same pattern. In other words, do not tie into an overloaded system or a system susceptible to line surges with the very device you are depending upon to control the foundation of the operation.

The second condition which has hampered instrumentation and automation has been the resistance of operators to admit that controls can do a better job of operating than can manual operation.

The first problem undoubtedly can be rectified by an understanding of the problems by the engineering and maintenance personnel of the plant or company. The second problem is a little more complex and, we believe, must be sold by management to the entire operating personnel of the organization.

It might appear that instrumentation and automation are suggested and designed for the sole purpose of eliminating labor. This, of course, is not true, since this type of control in the field of evaporation and drying is primarily to create a stable condition upon which better products and better production may be achieved, through research and development. It is true, however, that relatively few machines, manually controlled, give maximum production, minimum maintenance, and consistently better quality.

Central development. Each group in the development field is harassed by the same question: Will the results compensate for the money and effort expended to achieve the project? This question alone places a few basic problems at the head of the list. These problems are: (1) A marked reduction in floor space required for the units. (2) Efforts to make the unit self-sufficient, so that additional boilers or expensive heat transfer equipment is not necessary when a new installation is made. (3) Improvement in atomization. (4) Improved techniques in handling air and air filtration. And (5) greater versatility of the machine for new products by a wide variation in drying techniques. Of course, each of these basic subjects presents a host of minor items which are connected with them and, by the same reasoning, an over-all effort must be made to permit existing machinery to be adapted to new developments with a minimum of alteration. Many of these problems sound familiar and probably have been dismissed as adequately solved. This is not the case. For instance, most new products depend on consistency in particle size, heat treatment, and texture. Without precise control of the basic product, new products are not likely to be successful.

New products. New products depend more and more upon multistage processing techniques. Due to this, product research has

already dictated the approach we must take to the five major development problems.

In discussing the basic problems outlined, it becomes self-evident that a new machine which is designed to fulfill those requirements must also be superior in operation, due to the fact that many new products will be more difficult to handle with respect to vapor separation, heat treatment, and air filtration. New products seem remote in some respects, in relation to the drying operation in this particular area. However, it is of interest to note that in certain parts of the United States specialty items make up a great percentage of the over-all drying operation. This fact is due to the population increase and the ever-rising cost of raw products. Many plants in these areas have found it impossible to compete with the Midwest market on nonfat powder. If these plants can survive this predicament, it then becomes evident that many plants, regardless of the area, could take advantage of better profits during certain seasons of the year by producing a greater variety of dried products.

Space requirements. With this basis upon which to work, we might point out that the problem of reducing floor space required, or to put it in terms of cubic space required for the housing of the units, has long been a goal. For example, 10 yr. ago a unit producing 1,700 lb. of dried product per hour required 32% more heat release. A reduction of approximately 20% in the space required for the evaporator, and 26% reduction in space required for the dryer, have been achieved to date.

Certain minimum requirements are inherent in the design of any unit and, for this reason, a radical change in thinking does accompany work projected along this line. A good example of one of the minimum requirements is the general rule that a powder particle must travel approximately 42 in., under normal drying conditions, before it is self-sufficient. Normal drying conditions mean an average temperature of 340° F. inlet air, and a total solids content of 43%. Self-sufficient means that a particle is dried to a point that it will not readily adhere to a metal surface.

In an effort to make units self-sufficient, the direct gas-fired burner has probably been the most notable of all development work of recent years. These units have been quite successful, for the most part, and have been an economical step in the right direction. The gas burner requires less B.t.u. input to produce a given air temperature, by eliminating the heat losses in transfer at the boiler and heating coils.

Atomization. In expressing an opinion on atomization and efforts toward improving it, we must recognize that it is one of the most highly controversial subjects and has had much research attention. This is not only true in the food industry but also in many other industries. Two methods of atomization which have not been particularly popular in the food industry are atomization by steam and atomization by air pressure. Both offer some very interesting results, and it seems certain that either, or perhaps both, will be used to a greater extent in the future.

Air filtration. To improve methods in air handling and air filtration takes us back to the basic instrument problem for air being handled prior to the drying operation. As machines increase in size and productivity, they require more air at higher velocity. This requirement usually dictates that the air will be taken from an outside source. To stabilize the process, temperature must be taken into consideration and result in modulating machinery to correct for the change in density of the inlet air.

The stratification of hot air is another problem in this category and may be approached from at least two directions: (1) By instruments, which would vary certain conditions within the unit. (2) By having the air-heating system broken into segments which may be adjusted independently.

Air filtration is a highly controversial subject, but becomes an important item as the speed of the unit increases. A wide range in density and sensitivity of the product can also be expected from the list of new products which will place additional demands upon the system. Total area can be kept within reasonable limits in filtration systems if good air purging and agitation can be effected with these systems. Current efforts along these lines would seem to indicate that a better job with less material can be accomplished.

Versatility of the machine is, in some respects, of course, a result of the four items expressed in the foregoing. A number of products now being sold on the market require great moisture variation. For instance, powder being taken from the primary dryer can and does vary from 2% to as high as 18%. Obviously, these conditions dictate a wide range of drying temperatures, air velocity, and efficient conveying equipment.

In an effort to achieve the major subjects, a number of auxiliary items have been produced, as well as many new operating techniques. Among these are the direct-injection steam heater and heating by steam absorption. These two should not be confused. Both heat the product by the direct contact of steam. The difference between the two is the fact that direct-injection steam heaters usually inject the steam under high velocity, and this velocity is used to help offset excessive back pressure. The other type heater is a vessel in which the product is submitted to a given steam pressure. Each type has its specific advantages and disadvantages. However, the ability of either type to attain a temperature of 300°F. during con-

tinuous operation allows a number of relatively new processes to become a reality. The heaters also are compatible to evaporator operation and usually are located either ahead of the first effect as a secondary preheater or, in some instances, follow the product removal pump of the evaporator. It might appear that the addition of steam injected into the product would do little more than add dilution. This condition is offset by additional flash created within the evaporator. The use of these units also permits shorter holding time prior to evaporation, and both have played an important part in the production of aseptic products. Some criticism has been drawn toward this type of heating, due to contamination by direct contact with steam. It is well known that one of the greatest hazards is the use of "tannic" acid (or tannin) in boiler compounds. This should be kept in mind, of course, and eliminated from any system using the heaters. One of the better safeguards for this system is a conductivity cell within the steam line, which will register the conductivity of the steam on a chart and, at the same time, if the conductivity rises above that of normal steam, automatically shut the system down.

Another item in relation to evaporation is the restreaming of a triple-effect evaporator to control heat treatment, thus changing a normally high-heat evaporator into a typical low-heat operation. The evaporators themselves are also going through an evolution of combining nozzles, chests, separators, and so forth, to reduce space requirements and at the same time improve separation. It might also be mentioned that the falling film or downblast type evaporator is receiving more interest from the food industry.

One item in connection with dryers which is relatively new is the fluid drive, high-pressure pump, requiring no circulation to modulate pressure. The unit also is adaptable for automatic temperature control of the dryer. This type of pump was produced primarily for products which can not tolerate recirculation. However, it is adaptable to nearly any type of drying operation.

Vibrating tray dryers have been revived and are used for many secondary drying and cooling problems. One of their chief merits is to dry a given product in a given length of time, without serious mechanical damage to the product. By this ability, the correlation of time, temperature, and moisture can be adjusted much more readily than in some of the other types of secondary drying equipment.

Multistage drying seems to be more and more important, due to its ability to change the structure of a product by first shifting the lactose balance (in a milk product) and to produce a crystalline type produce under high moisture conditions in the primary drying equipment. When this conversion is accomplished, the powder is relatively free from stickiness at moistures as high as 18%. Of course, secondary drying plays a most important part in this type of operation.

Obviously, the items mentioned here are but a token of development work in connection with evaporators and dryers within the recent past. There is very little new basic thinking in the development field. This is better illustrated by the fact that over 100 patents have been issued in connection with the two basic machines and which are in connection with food processing. Of course, this does not include the multitude of patents issued in connection with auxiliary equipment. Much of the information contained in the patents mentioned has been buried, for one reason or another. However, the basis for must future development already has been firmly established. With fixed conditions established within the evaporator and dryer, development work will move ahead rapidly, with a minimum of mechanical difficulty.

It is reasonable to believe that, in the near future, a recording instrument will be located in the superintendent's or manager's office which will give him a look into nearly every process within his plant and will, in effect, bring into profitable service those items which have been held back, awaiting the right time.

OUR ASSOCIATION

THE 54TH ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION JUNE 15–17, 1959

H. F. JUDKINS, Secretary-Treasurer

The 54th Annual Meeting of the American Dairy Science Association at the University of Illinois, June 15–17, 1959, was one of the largest ever held. No general session was held this year to open the meetings; instead, the section programs started on Monday morning, June 15.

The annual Past Presidents' meeting and dinner was held from 4:30 to 6:30 P.M., June 15, attended by 18 Past Presidents and members of the Board of Directors. A discussion was programmed on Suggested Courses of Action for the Board of Directors of the American Dairy Science Association, which will (a) Attract and hold the interest of more members from industry. An analysis of membership, past, present, and future—by H. F. Judkins; (b) Increase the percentage of papers in the JOURNAL OF DAIRY SCIENCE on processing and management problems. A discussion by P. H. TRACY; (c) Coordinate more closely the research programs in colleges with industrial needs. A talk by E. H. Parfitt.

The General Session, devoted to Association affairs, was held Tuesday, June 16, President Turk presiding. It was emphasized that the American Dairy Science Association was founded at the University of Illinois in 1906. Dr. Turk showed a slide of the 17 charter members who were present at this meeting. The three living charter members are Dr. E. S. Guthrie of Ithaca, New York, Dr. O. F. Hunziker of LaGrange, Illinois, and Dr. C. C. Hayden of Monroe, Virginia. The letter two are in ill health, but Dr. Guthrie was present and occupied a seat of honor on the platform.

The formation of the Association in 1906 was initiated by Professor W. J. Fraser, at that time Head of the Dairy Husbandry Department at the University of Illinois. Mrs. Fraser still lives in Urbana. She occupied a seat of honor on the platform and was presented to the membership. In response to the receipt of some flowers which were sent to her, the following letter was received:

> With deep appreciation I accept your gift of those lovely roses as a beautiful and wonderful tribute to my husband.
>
> It was such an honor and inspiration to be in your midst and I consider it one of my most treasured experiences.
>
> Who could dream what a tremendous force would grow from the banding together of that little group of seventeen? And the end is not yet. It is said "Without a vision the people perish." Who can deny that this little group had it? I bespeak for you greater and greater expansion as the years go on. My interest and good wishes are always with you.
>
> I cannot adequately express my heartfelt gratitude and appreciation for all that the gift of those lovely roses means to me.
>
> Sincerely,
> MRS. WILBER FRASER



Mrs. Wilbur J. Fraser, wife of the late Wilbur J. Fraser, distinguished Professor of Dairy Husbandry at the University of Illinois and prominent founder of the American Dairy Science Association.

Dr. E. S. Guthrie, distinguished Professor Emeritus of Dairy Industry at Cornell University, and one of the three living charter members of the Association.

Dr. G. W. Salisbury, head of the Department of Dairy Science, introduced Dr. David D. Henry, President of the University of Illinois, who welcomed the Association membership to the University.

Remarks of Welcome by President David D. Henry

It is a great personal pleasure to meet and greet the American Dairy Science Association. The planning for this meeting has gone on for over four years. We hope the consummation of the planning will justify every detail.

As you know, from repetition, the University of Illinois is proud of its association with the American Dairy Science Association, of the

fact that the founding was on this campus in 1906, and of the leadership of charter members, and others through the years. That pride in association stems from our appreciation of Dairy Science in our University as a field of study, and of Dairy Science as in Industry. The University of Illinois is a center for improved production, discovery and education.

Our interest in the Association goes beyond these reflections of ourselves. The American Dairy Science Association brings together education, research interests, and industry. This interdependence is obvious but not recognized frequently enough.

In meeting on land-grant campuses, you appropriately relate these institutions to your industry. Industries dependent upon the products of land-grant colleges do not often enough acknowledge this dependence, or as much as might be expected show interest in investment in the future, in gifts, or in aggressive support for the future.

Unless the University supply line keeps pace with the developments in the economic life of the State, the ultimate growth in the economy will be less than anticipated. The University is at the heart of the supply of trained personnel, of specialists for management and operation, and of research for the advancement of basic knowledge and the application of that knowledge through new processes and new procedures.

It is a great problem in a growing and prosperous state to have the public understand the relationship between education and prosperous growth. We can not have the one without the other. There can not be an increase in income, or an increase in the capital structure of business, unless these increases are accompanied by an increase in the cost of government activities which are related to that growth. New government revenue must be seen as a natural part of the growth of the economic structure itself.

In the fall of 1961 will begin the centennial year of the Land Grant Act. There will be appropriate observances.

The Land Grant Institution maintains its traditional role of service to agriculture, but the spirit of the original enterprise now extends to all of American life, and to all of the disciplines. Occasionally, a voice in agriculture regrets this. Most do not, as the interdependence of modern life is recognized.

The spirit of the land-grant institution is still a dominant note in higher education: wide inquiry without provincialism, a concern with the general welfare without regionalism, and adaptability without parochialism or restraint from the traditional.

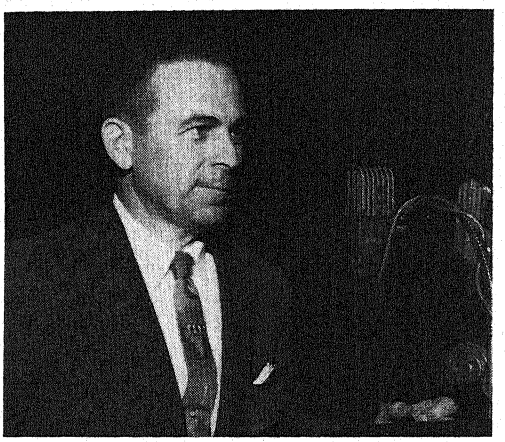
All Land Grant institutions welcome your interest and support.

Perhaps I should have used my time to talk about the University of Illinois, the three campuses, new programs, and new challenges. We assume you will be indoctrinated more subtly. We hope you will like what you see. We thank you for the inspiration of your being here.

In traditional and in scholarly fashion, Dr. K. L. Turk presented the annual Presidential report to the Association membership.

PRESIDENTIAL REPORT

K. L. Turk
Department of Animal Husbandry,
Cornell University, Ithaca, New York



President K. L. Turk.

President Henry, on behalf of the members and officers of the American Dairy Science Association I express to you our sincere thanks for your invitation to meet on your fine campus. We appreciate the excellent cooperation given us by Professor Salisbury and all other members of the Illinois staff. Plans were carefully made; nothing has been left undone. We know and appreciate the amount of time and labor the host institution puts into an annual meeting like this one. It will go down in history as our largest meeting to date and also one of the most successful. This has been a pleasant and profitable experience for all of us.

It is traditional for the President of the American Dairy Science Association to give a "Presidential Address." Last year, however, President Josephson preferred to designate his address as a "Report to the Association." Since this comes during our annual business session, I shall follow Past-President Josephson's example.

The success or failure of organizations like A.D.S.A. depends largely upon the membership. We are too large a group to be administered completely by the officers and Board of Directors. Over the years, therefore, an effective committee organization has been developed, not only in the main body of the organization, but in each of the three main sections.

We have a total of more than 200 members serving our Association on the many standing and special committees. I want to thank all of them for their conscientious efforts toward the betterment of our Association.

Special mention should be made of the excellent work and unselfish devotion of our Secretary-Treasurer, Dr. H. F. Judkins. In his position, he is a key person who really makes our Association move. No one is more appreciative of this than your President.

A definite attempt has been made this year to create more interest and participation in the Association by our members from industry. Almost one-fourth of the membership of all of our committees comprises persons working in in the industries and in dairy organizations. Your president-elect, Dr. A. C. Fay, is keenly interested in ways and means of bringing about more participation in the affairs of our Association by industry members.

Your officers and directors and members of committees devote much time and energy to the functioning processes of this Association. Our constant goals are a Journal that is as useful as possible; annual meetings that are attractive and valuable for all segments of our membership; and the active interest and participation of our members — those in industry, along with those from the academic fields.

Now, a few brief comments on the work of some of the committees:

PROGRAM. Your program committees for many years have given careful thought and attention and many hours of labor to our annual meeting programs. The committee this year, under the able leadership of Dr. Fred J. Babel of Purdue, has done a fine job, and I know you will agree. The committee meets in the fall in Chicago and spends a couple of days making out the general program. Much time is spent in discussing ways and means of maintaining and improving the quality of our scientific presentations. If improvement is needed in our papers, it must be brought about by you; if papers are to be screened more rigidly, it must be done in the departments or laboratories from which they come. I am confident that if all of us give more consideration to the quality of our papers and abstracts, it will result in more useful and valuable meetings of our Association.

ASSOCIATION POLICY. A new standing committee, known as the Association Policy Committee, was established by the Executive Board and approved by the membership in 1957. Under the able chairmanship of Past-President Walter V. Price of Wisconsin, this committee, composed largely of senior citizens, is contributing much to our long-range policy matters. In addition, it considers specific policy questions that may be referred to it by the Executive Board.

As the last item on our agenda this morning, Dr. Price will present suggestions concerning the future of our Association. We know this will stimulate much thought, and, we hope, comments from you.

STUDENT AFFILIATE. Our future membership will come largely from undergraduate and graduate students in colleges and universities. One of our most significant committees, therefore, is that of Student Affiliate, under the chairmanship of Professor W. W. Snyder of Michigan State University.

A new feature this year is a $100 cash award given by the Association for the outstanding student affiliate chapter. We now have 41 chapters at our colleges and universities, with a total membership of more than 700. You may want to look over the scrapbook at registration headquarters. Another innovation is the graduate student scientfic presentation award of $100. This program is being conducted in the production section on a trial basis this year. We hope it will become a regular award both in the production and in the manufacturing sections.

MEMBERSHIP. Under the chairmanship of J. B. Frye, Jr., of Louisiana State University, this committee has done another fine job. The goal of 2,500 members has been exceeded. We still have the perennial problem involving those persons who fail to renew their memberships before January 1 of each year. This creates a tremendous load for our Secretary-Treasurer. We are happy to get many new members each year, but we are always sorry to lose members.

PUBLIC HEALTH. This relatively new committee under the able leadership of Dr. L. A. Black has been very active in timely studies of problems relating to public health. For example, reports on Milk Regulations, Dairy Feeds and Drug Additives, and 3-A Sanitary Standards were published in the April, 1959, JOURNAL OF DAIRY SCIENCE. Other reports, recently referred to the Executive Board for review, deal with Radiation, Foreign Substances in Milk and Milk Products, Antibiotics, and Some Current Aspects of Animal Disease Control.

It is exceedingly important that steps be taken to provide better and more accurate public understanding regarding the occurrence and significance of strontium^{90}, pesticide and antibiotic residues, and food additives. The occurrence of strontium^{90} is not a problem peculiar to milk, and much harm can be done to our industry through misinterpretation by the public press. This is an area in which much research is needed, to develop new knowledge and facts for the best interests of public health and for the dairy industry.

EDUCATION. Last year, the special Committee on Education became a standing committee of the Association. Under the chairmanship of Professor J. T. Lazar, Jr., of Clemson College, this committee planned and conducted an excellent panel discussion yesterday concerning enrollment problems in dairy science.

We agree wholeheartedly with the recommendation of this committee that education is important enough to have the attention of a majority of the members of the Association. Our job of educating and training young people

for the dairy industry must have strong interest and support from the dairy industry, from other related fields, from dairy extension, from research laboratories, and from dairy farmers. At the same time, those of us in the colleges must always assume our full responsibilities to provide high-quality education in dairy science.

A special committee of the Association has been working long and diligently on the preparation of a Dairy Industry Plant Training Manual, and I take this opportunity to thank Professor H. B. Henderson and his committee for completing their task. A final draft was submitted to the Secretary on April 16 for delivery to the printer. Favorable comments on this manual have been received. We hope it will be used extensively by the industry and that it will prove beneficial to college graduates in dairy technology.

JOURNAL OF DAIRY SCIENCE and JOURNAL MANAGEMENT. Our Journal continues to grow under the expert editorship of Dr. E. O. Herreid. This is a time-consuming and trying job, and we are all grateful to him. Excellent progress has been made in getting publication and delivery of the Journal moved up to more satisfactory dates. For this cooperation, we are grateful to the Garrard Press. Special mention also should be made here of the Journal Management Committee under the chairmanship of Dr. D. H. Jacobsen of Chicago. Since January 1 of this year, you have been receiving Dairy Science Abstracts under the terms of our arrangement with the Commonwealth Agricultural Bureau in England. These abstracts should prove popular with our membership.

Let's turn now to some of the challenges that face us.

Tremendous changes have taken place in the progresive development of the dairy industry to its present place of importance. We are proud of the important role the American Dairy Science Association has played in this progress through education, including both resident teaching and extension, and through basic and applied research provided by its active members.

Without the scientific contributions of chemists, biochemists, physicists, bacteriologists, physiologists, nutritionists, dairy technologists, geneticists, and dairy husbandmen, it would not be possible for modern dairying to exist in its present form. Modern dairying is an industry based on scientific discoveries and inventions. If it is to continue to move forward successfully, our industry must depend upon higher-quality education, well-trained scientists and teachers, improved public relations, and continuous fundamental research.

Rapid changes and adjustments continue to take place on the nation's dairy farms. The number of farms is decreasing at a rate of about 4% per year; farms are increasing in size with more cows per farm and are becoming more highly mechanized and more specialized; milk production per cow continues to rise; and output per man has more than doubled. Will there be a leveling-out point on some of these changes? If so, when?

One thing is sure. We will continue to have change and adjustment. We will have fewer farmers, but of necessity they must be better farmers. They must be more highly educated and more skilled, with the ability to apply the results of research and modern technology. As Charles Dickens once said: "The best acreage for a farmer to cultivate lies within the ring fence of his skull."

We have been greatly concerned about decreasing enrollments in agriculture in our colleges, especially in dairy industry and technology. If this trend isn't reversed soon, will we have enough educated and trained persons to assume positions of leadership in our industry?

I should like to make a special plea that all of us in the educational field take a renewed and vital interest in our teaching responsibilities. Let's give teaching more emphasis in our graduate schools. Great teachers are needed to attract and inspire good students.

I particularly liked Dr. C. F. Huffman's discussion of education in his presidential address in 1957 at Oklahoma State University: "Most of us have had the experience of finding subject matter come alive because of the presence of a great teacher. The influence of the teacher is not necessarily determined by his subject matter, but by his skillful handling of it. What we need in our colleges and universities are teachers of professional and specialized subjects who see their subject matter in relation to the whole field of modern learning and modern civilization. We need teachers who see their subject matter not only as a means for developing techniques and skill, vitally important as they are, but also as a means for opening vistas which will challenge and inspire the students' imaginations."

Let's reward good teaching in our colleges and universities. Outstanding teaching needs to be given more consideration in promotions and professional advancement. The idea is all too prevalent that the only way to obtain professional advancement is through published papers.

Science education has been given a tremendous boost in recent years. This is excellent and most timely. In our enthusiasm for basic sciences, however, let's not forget a liberal education—a fundamental education, including the humanities.

And in dairying, I hope we won't forget that our enormous industry hinges on the dairy cow. Too often it is difficult to find a prospective staff member who is willing to teach dairy husbandry or to conduct research with dairy cows.

I make this plea for better teaching because I feel that teaching has been neglected far too long. Have we tended to overlook the fact that

the foremost function of a college or university is to teach students? Too many of us in college work simply take our teaching for granted. I am condent that if more of us would devote the same time and effort and sincere interest to teaching that we give to research, we would attract more students to dairying.

At the same time, I hope you will not feel that I underestimate the importance of basic and applied research. In this rapidly changing era, research is essential for survival. Our future depends upon keeping dairy science dynamic and on the move. We must constantly make strides in more efficient advancement in all areas of dairy science, including production, manufacturing, and marketing.

Dairying is now suffering a major blow from several angles, which affects the consumption of our products. We have a calorie-conscious consuming public. With the antifat propaganda, resistance to animal fats continues. There is much talk—some of it without sufficient scientific basis—of relationships of animal fats, including milk fat containing cholesterol, to arteriosclerosis. Newspapers have been quick to publish articles on the dangers of strontium^{90}, pesticide residues, and antibioties in milk. Some recent news items have reported opposition to the use of dairy products for elderly persons.

These problems point up the job we have in public education with milk producers and handlers, as well as the consumer. Likewise, they focus the need for increased basic research in these areas. As Thomas A. Edison once said, "Nothing runs by itself except downhill." We may have an uphill struggle, but we are equal to it.

We all recognize the achievements of the American Dairy Association in publicizing the importance of milk and dairy products and the profound influence this program is having on our industry. But we must assume our responsibilities in marketing, too.

"Nothing in education is so astonishing as the amount of ignorance it accumulates in the form of inert facts." This quotation from the *Education of Henry Adams* emphasizes the need for the scientist to interpret his results and make them available to people who are in a position to use them. The job of the researcher is not finished until the results of his fertile brain are put to work for the good of mankind.

I am confident we will use our scientific accomplishments and trained manpower to the fullest extent to build a bigger and more important dairy industry in the future. We can not miss if we hitch our star to improved education and training methods, dynamic extension education and public relations, fundamental and applied research, and thorough cooperative efforts among all facets of the dairy industry.

Thank you for the honor and privilege you have given me as your President this year. The time has passed too quickly for the accomplishment of all that we had hoped. Ours is a dynamic Association and there always will be constructive work for future committees, Executive Boards, and Presidents. We look forward to many challenging years ahead.

AWARDS PROGRAM

The Awards Program and the installation of officers was held on the evening of June 16 in the University Auditorium. This was a very distinguished affair and the feature event of the annual meeting. Professor Stephen Farish, baritone, accompanied by his wife, Anna, entertained with selections from popular musical shows. Dr. Paul H. Tracy of the Department of Food Technology presided over the entertainment part of the program. President K. L. Turk presided over the awards ceremonies which consisted of citing and presenting the honored members for their individual awards.

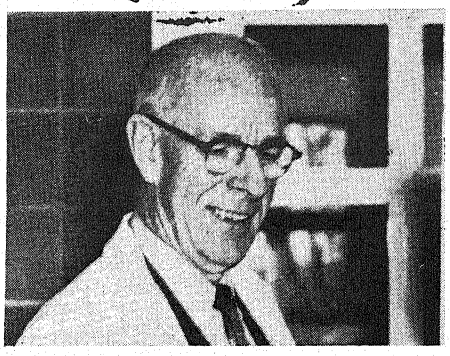
Citation by Dr. G. M. Trout for the Recipient of The American Dairy Science Association Teaching Award in Dairy Manufacturing for 1959

The Executive Board, American Dairy Science Association, in session June, 1954, approved the Teaching Award Committee's report. This report provided for special recognition to outstanding teachers in dairy science. Since then an award of $1,000 and a plaque have been presented each year to outstanding dairy science teachers. These awards alternate yearly between the field of dairy manufacturing and that of dairy production.

The teacher selected this year for special recognition by the American Dairy Science Association has achieved an enviable teaching record in dairy manufacturing, not only in the college classroom but in conferences throughout the state, nation, and foreign countries, as well. Despite the passing of time, he has continued to expound dairy science with the conviction and



Mr. C. V. Christensen, representing the Milk Industry Foundation, presenting the American Dairy Science Association Teaching Award to Dr. H. A. Bendixen, with Dr. G. M. Trout, who read the citation.



Dr. H. A. Bendixen

enthusiasm of an evangelist. Scores of former students now in responsible positions throughout the United States attest his efficacy as a teacher.

His former dean, after many years of observation, describes him as a born teacher. He stated further,

> "His courses were well organized and his teaching was very methodical and exact. He expected and secured from his students complete compliance and exactness. No complimentary marks or grades were given. The student received just exactly what he earned. While some complained at times, they respected him for his firmness and justice."

Briefly summarized, the candidate has been a painstaking teacher, a strict taskmaster, a confiding counsellor, and a friend of every student on whom his book was never closed. In addition, he has achieved distinction as a student employment agent, a repeatedly winning dairy products-judging team coach, a dairy manufacturing conference and dairy products-clinic organizer, a dairy conference lecturer, a prolific writer, a constantly sought dairy industry consultant, and a liaison agent between education and the dairy industry.

The Teaching Award selectee for 1959 was educated both in Europe and America. Born in Hamburg, Germany, March 6, 1894, he emigrated to America of his own volition in 1912. He attended the College of Commerce, Waterloo, Iowa, 1913-1914. Working his way through Iowa State College, he graduated, 1919, magna cum laude, with the B.S. degree in dairy industry. From the same institution he received the M.S. and Ph.D. degrees in dairy bacteriology in 1927 and in 1937, respectively. From 1920 to 1927 he was assistant professor and associate professor at the University of Idaho and associate professor and professor at State College of Washington from 1927 to the present. Not only has he been honored by membership in the honor societies of Sigma Xi, Phi Kappa Phi (of which he is now president), Gamma Sigma Delta, and Sigma Delta Chi, but repeatedly in a big way he has been honored by his former students and the dairy industry of the state he has served so well.

The American Dairy Science Association wishes to honor this evening with the teaching award in dairy manufacturers one of its tireless workers and outstanding leaders, a past president of the Association—Hans Adolf Bendixen.

President Turk, on behalf of the Teaching Award Committee of the American Dairy Science Association, we are pleased to present to you Dr. Hans Adolf Bendixen, Professor, Washington State College, who has been selected to receive the Teaching Award for 1959.

Citation by Professor W. R. Van Sant for the Recipient of the DeLaval Extension Award for 1959

The man selected to receive the 1959 DeLaval Extension Award was born 54 years ago in Chicago, Illinois. He began his college training in 1926 by attending Pennsylvania State College for two years. He transferred to Cornell University and received his Bachelor of Science degree in 1930, graduating second in his class. The following year he was an assistant in Dairy Husbandry at Cornell University and completed the work for his Master of Science degree.

After receiving his Master of Science degree, he started farming on his own; first, on the home farm and later on the farm he and his wife purchased. He is now the Extension Project Leader of Dairy and Animal Husbandry at the same institution where he began his Extension career in 1937.

His training and personal interests in breeding dairy cattle qualified him for leadership in the extension dairy breeding program. His experience while farming, and as president of the first artificial breeding association in the state, were of great benefit to him in planning and working out the artificial insemination program. He has been an outstanding leader in developing training schools for artificial breeding technicians. He assisted in the organizaton of the National Association of Artificial Breeding Cooperatives.

In addition to the leadership that he has given to several projects in dairy cattle breeding, he has participated in all phases of Dairy Extension. He regularly conducts feeding and feed production schools and health meetings and gives active supervision to Dairy Herd Improvement Associations.

He is a member of the American Dairy Science Association and has served on, and has been chairman of, the breeding, breed relations, and type committees. He has been secretary, vice-chairman, and chairman of the Extension Section. He is also a member of Epsilon Sigma Phi and the Holstein-Friesian Association of America. In 1955, he was awarded the Superior Service Award by the Secretary of Agriculture for outstanding work with the dairymen of his state.

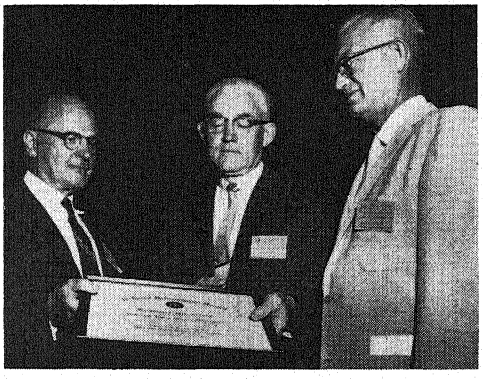
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Ray Albrectsen

This man is an unusually well-balanced Extension Specialist and is respected by his coworkers, county agents, and dairymen. His clear and forceful presentation of subject matter has created a demand for his services throughout the country.

On behalf of the DeLaval Extension Award Committee of the American Dairy Science Association, I am honored to present Professor Raymond Albrectsen, New York State College of Agriculture, Cornell University, and Extension Project Leader of Dairy and Animal Husbandry, to receive the DeLaval Extension Dairyman Award.



Mr. W. A. McGill of the DeLaval Company presenting the DeLaval Extension Dairyman Award to Professor Ray Albrectsen, with Professor W. R. Van Sant, who read the citation.

Citation by Mr. R. E. Ward for the Recipient of the American Feed Manufacturers Award for 1959

The American Feed Manufacturers' Award is donated by the sponsor and presented each year to encourage research in dairy cattle nutrition. The man selected for the 1959 Award was judged by a committee of the Association to have made the most significant contributions to dairy cattle nutrition during the past 3-yr. period.

During this time, the recipient has been senior author of seven journal articles and coauthor of four. He was author of six published abstracts and coauthor of four. Among his many contributions, the most noteworthy are his studies on calcium and phosphorus metabolism in cattle fed large doses of vitamin D for the prevention of milk fever, and his studies on early rumen function of the calf and the high roughage system of calf feeding. He has given special attention to rumen function and has used P^{32}-tagged erythrocytes in measuring gastrosplenic blood flow and volatile fatty acid absorption from the rumen. Other research he has done includes protein metabolism in the dairy cow, bloat, occurrence and alleviation of anemia in calves, and goitrogenic activity of legume silages. These are truly notable contributions in this short time.



Dr. Harry R. Conrad

The recipient was born on a farm near Burlington, Kentucky, on October 3, 1925. He attended his state university, where in 1948 he was the outstanding male graduate and received the Sullivan Medallion in recognition of his attainment.



Mr. R. E. Ward presenting the American Feed Manufacturers Award to Dr. Harry Russell Conrad, with Dr. J. K. Loosli who read the citation.

In 1949, he received the Master of Science degree and in 1952 the Doctor of Philosophy degree in Dairy Science, both from The Ohio State University. He is a member of Alpha Zeta and Sigma Xi. Since completion of his doctorate, he has served as Assistant Professor on the staff of the Ohio Agricultural Experiment Station at Wooster, Ohio.

On behalf of the selection committee and the Association, it is my pleasure to ask Dr. Harry Russell Conrad to come forward to receive the 1959 American Feed Manufacturers' Award.

Citation by Dr. G. H. Hartman for the Recipient of the Borden Award in Dairy Manufacturing for 1959

The man selected to receive the Borden Award in Dairy Manufacturing was born in Maryland. He obtained the Bachelor's and Master's Degrees from the University of Maryland, and the Doctor's Degree in 1940 from Cornell University. For 6 yr. he was Assistant Chief Bacteriologist at the National Dairy Research Laboratories. In 1947, he went into college work and is now a Professor of Dairy Bacteriology. He is engaged in teaching and supervising graduate student thesis research, and about half of his time is devoted to research in dairy bacteriology. In addition, he has been a consultant to the Dairy Industry and to the U.S. Public Health Service. His most fundamental research contributions have been in the field of nutrition of bacteria, particularly on the application of growth stimulants for culture of microorganisms. Notable, too, is his work on the effects of lipasase activity and the products of lipolysis on microorganisms in milk, and work on basic time-temperature combinations for pasteurization. He also has made worthy contributions to the development of standards for detergent-sanitizers for farm use.



Dr. Marvin L. Speck

The recipient of the 1959 Borden Award in Dairy Manufacturing is a member of, and has held offices in, the Society of American Bacteriologists, North Carolina Society of Bacteriologists, International Association of Milk and Food Sanitarians and, of course, the American Dairy Science Association. From 1951 to 1953 he was Chairman of the Committee on Standard Plate Count, and since 1953 he has been an Editorial Board Member of the JOURNAL OF DAIRY SCIENCE.

I am very pleased to name as recipient of the Borden Award in Dairy Manufacturing one who is well-known to this group, Dr. Marvin L. Speck, Professor of Dairy Bacteriology, North Carolina State College.



Mr. W. N. Waterstreet of the Borden Company presenting the Borden Award in Dairy Manufacturing to Dr. Marvin L. Speck, with Dr. G. H. Hartman, who read the citation.

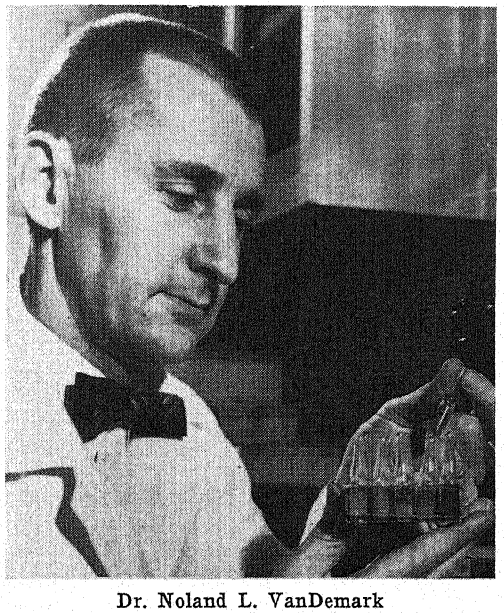
Citation by Dr. H. Ernest Bechtel for the Recipient of the Borden Award in Dairy Production for 1959

This awardee was born July 6, 1919, near Columbus Grove, Ohio, and was raised on a dairy and livestock farm. After receiving his elementary and high-school training in his home community, he majored in animal science at The Ohio State University and received the B.S. degree in agriculture in 1941. He stayed on, however, as a graduate assistant and received the M.S. degree in 1942—after also having spent several months in establishing a vitamin research laboratory for the Division of Plant Industry of the Ohio Department of

Agriculture. After two more years as a graduate assistant in animal husbandry at Cornell University, he entered the Army in 1944 as a private, was commissioned a Second Lieutenant the following June, trained and served as a counter-intelligence agent in Germany until July, 1946, and spent several months organizing and initiating an artificial breeding program for cattle in Austria before resuming graduate studies in early 1948. He majored in animal husbandry, with minors in animal nutrition and animal physiology, and received the Ph.D. degree at Cornell University in September, 1948. He has been on the University of Illinois staff since that time in dairy cattle physiology—first as Assistant Professor, then as Associate Professor and, since 1955, as Professor. On campus, he is outstanding as a teacher of undergraduate and graduate students, and his classes are filled to capacity. He also is in demand here and abroad as a counselor and speaker, and has been honored with special invitations from Austria, Copenhagen, and London. Along with these accomplishments as a teacher and speaker, his work is characterized by continuing scholarly studies in his chosen field of bovine reproductive physiology.

His studies on preserving spermatozoa at room temperature led to fundamental knowledge on the metabolic rates of spermatozoa as influenced by the partial pressure of carbon dioxide in the surrounding gas phase, with far-reaching implications already evident in the field of human medicine, where the idea of carbon dioxide inhibition of cell metabolism provides the basis for a new theory on the etiology of cancer.

Our Selection Committee is sincerely proud and satisfied to present Dr. Noland Leroy VanDemark of the Department of Dairy Science at the University of Illinois as the 1959 recipient of the Borden Award in Dairy Production of the American Dairy Science Association.



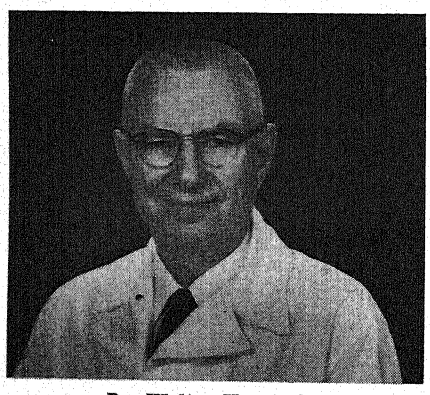
Dr. Noland L. VanDemark



Mr. W. N. Waterstreet of the Borden Company, presenting the Borden Award in Dairy Production to Dr. Noland Leroy VanDemark, with J. F. Cavanaugh (center), who read the citation, and Dr. G. W. Salisbury.

Citation by Dr. B. H. Webb for the Recipient of the First Paul Lewis Award in Cheese Research in 1959

The candidate for the first Paul Lewis Award in Cheese Research began life in what was then the country's leading cheese-producing state, New York. He received three degrees from Cornell University, but seeming to sense a shift in cheese production, he went west to continue to work in the nation's largest cheese-producing state, Wisconsin. Here at the State University, for the last 31 yr., he has made outstanding contributions to cheese research and to teaching. Since this candidate received the Borden Award in Dairy Manufacturing from this Association within the last decade, research done only since then has been considered for the present Award. The work of the candidate has been of the highest caliber—voluminous, yet thorough. His studies on the milk used in cheese-making, its production, quality, and pasteurization have provided a better raw material for modern cheese manufacture. Research on cheese-making, including standardization of the Cheddar-making process; on curing; and on analytical procedures, including spectrophotometric studies in ripening cheese, has furnished the scientific basis necessary to the production of high-quality cheese. Dariworld, an entirely new variety of cheese, and cheese foods made from it, were developed under his guidance. His leadership, his cooperation with associates, and his teaching of students have been outstanding. Now it is indeed a pleasure, on behalf of the Paul Lewis Award Selection Committee, to present the 1959 candidate for the first Paul Lewis Award, the dairy industry's foremost authority on cheese, Dr. Walter Van Price.



Dr. Walter Van Price



Mr. Paul Halmbacher presenting the first Paul Lewis Award in cheese research to Dr. Walter Van Price, with Dr. B. H. Webb, who read the citation.

Citation by Dr. D. V. Josephson for the Recipient of the American Dairy Science Association's Award of Honor for 1959

The member of the Association selected for this year's Award of Honor may be considered as an elder statesman—one who has given a lifetime of service to the Dairy Industry as a university educator and administrator, and as a leader in industrial and civic affairs. The facets of this man's attention have been many, and his noteworthy contributions have been unusually numerous.

He is a native of Iowa and received all of his degrees, including the doctorate, at Iowa State College. For 37 yr., he has been associated with Montana State College, where he began as an Instructor in the Dairy Industry Department in 1922. He has held all of the academic ranks in the Department and for 27 yr. served as Professor and Department Head. For 7 yr. during this period (from 1948 to 1955) he served as Dean of the Graduate Division of the college.

In his responsibilities as staff member and administrator, the Award recipient contributed unusual strength to the Department through his teaching and, at one time or another, accepted the challenge of teaching each of the courses in Dairy Manufacturing. In addition, he organized and administered the State Dairy Industry Short Course for 21 yr., carried on an extensive educational program through regular extension releases to the Industry on technical and management problems, and initiated and carried forward a progressive program for quality improvement throughout the entire State of Montana. As a result of his many services, and because of his intimate knowledge of the various aspects of the Industry, his counsel has been sought by students, by faculty associates, and by industrial leaders. Although he has relinquished administrative responsibilities, he continues to serve the Montana State College and the State of Montana as Professor of Dairy Manufacturing.

A principal basis of his selection for this year's Award of Honor is his interest and participation in the activities of the American Dairy Science Association and the contributions he has made to its welfare. Until hampered by ill health, he was a regular attendant at the annual meetings. He served on the Executive Board in 1937 and 1938, was Vice-President in 1945 and President in 1946. He has served one term or more in important positions in the Western Section of A.D.S.A. with chairmanship in 1935 and 1936. He has exerted unusual influence in guiding the destiny of our Association as a result of his counsel with others who, in succeeding years, held positions of high responsibility in the organization.

This year's recipient played a major role in organizing the American Dairy Association of



Dr. John A. Nelson

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ALLAHABAD INSTITUTE



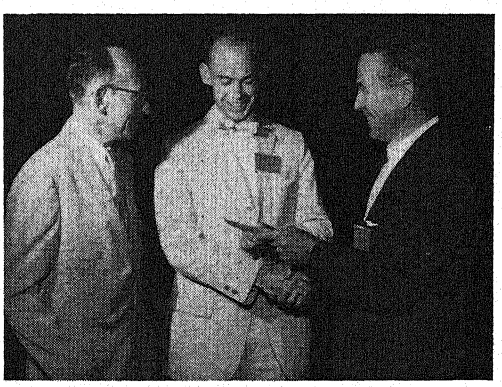
President K. L. Turk presenting the Award of Honor to Dr. John A. Nelson, with Dr. D. V. Josephson, who read the citation.

Montana and, for many years, filled important functions as a member of its Executive and Research Committees.

In civic work he has been an unusual force for good, and has filled key leadership positions in his church and as a member of the local Chamber of Commerce. He is married and has one son who is following in his father's footsteps in the field of Dairy Manufacturing.

Therefore, because of his many years of distinguished service to the entire Dairy Industry, and because of the numerous specific contributions to the American Dairy Science Association, Dr. John A. Nelson of Montana has been selected as the 1959 recipient of the highest recognition of our Association—the Award of Honor. President Turk, on behalf of the Honors Committee of the American Dairy Science Association, it is a pleasure to present Dr. Nelson.

Graduate Student Scientific Paper Presentation



President K. L. Turk presenting the American Dairy Science Scientific Paper Presentation Award for first place to J. B. Stone of Cornell University, with Professor W. W. Snyder, who read the citation.

Professor W. W. Snyder, Chairman of the Student Affiliate Committee, presented the first Graduate Student Scientific Paper Presentation Award of $100 and an appropriate certificate, donated by the Association, to J. B. Stone of Cornell University. This contest was conducted only in the Production Section. There were nine contestants. K. E. Ebner of the University of Illinois and E. W. Wickersham of Pennsylvania State University received Honorable Mention certificates.

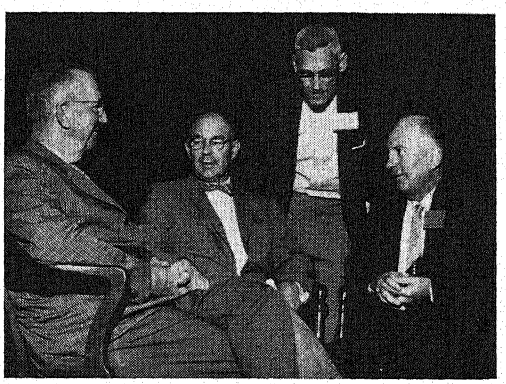
Student Affiliate Chapter Award for 1959

Professor W. W. Snyder made the presentation of $100 and a plaque, offered by the Association, to Louisiana State University, the winner of this year's Award. Eighteen chapters competed. Five received Certificates of Merit, seven Honorable Mention Certificates, and five Participation Certificates.



Left to right: Professor J. E. Johnston, Editor of the Student News Section of the Journal of Dairy Science, and Professor W. W. Snyder, Chairman of Student Affiliate Membership, visiting with Wayne Register, representing the Louisiana State University Dairy Science Club, winner of the first place in activities competition.

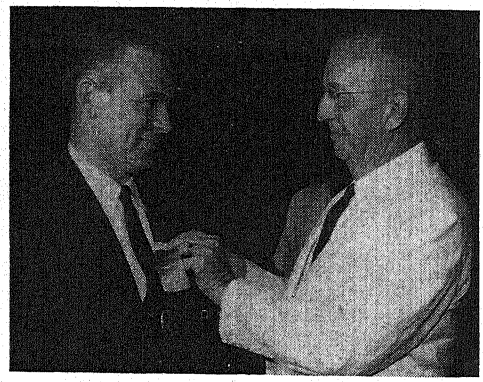
INSTALLATION OF OFFICERS



Newly elected officers (left to right) are: A. C. Fay, President; R. E. Hodgson, Vice-President; F. J. Doan, Director; and G. H. Wise, Director.

President K. L. Turk installed the following officers: Dr. G. H. Wise and Dr. F. J. Doan, newly elected Directors; Dr. R. E. Hodgson, Vice-President; and Dr. A. C. Fay, President.

Dr. Fay pinned a Past President's ribbon on Dr. Turk and adjourned the meeting.



President A. C. Fay pinning the Past-President's badge on Dr. K. L. Turk.

MINUTES OF MEETINGS OF THE EXECUTIVE BOARD FOR 1959

The Executive Board met at beautiful Allerton Park for three days. Sub-committees of the Board met on Friday afternoon, June 12, to consider the various committee reports which had been prepared and sent to them in advance by Vice-President Fay. This made it possible for the Executive Board to act more effectively. Nevertheless, there was a large volume of business, and the Executive Board met all day Saturday and Sunday, including evenings, with brief meetings on Tuesday morning, June 16, and Wednesday morning, June 17.

BOARD MEETING ACTIONS

I. *Minutes of 1958 Board Meeting.* It was voted that the minutes of the 1958 meeting presented by the Secretary be dispensed with.

II. *Secretary's report on interim matters, 1958-59.* Voted to confirm action of Board taken during the interim period.

(1) Secretary presented resolution from the National Dairy Council commending the Association for its activities in the dairy industry.

(2) $108 donated to the Dairy Remembrance Fund for purchase of a recruitment film "This Is the Dairy Industry" for some institutions not having funds to purchase same.

(3) Article II, Section II of the By-Laws changed so that the new officers will now be nominated by January 1 instead of April 1.

(4) Article III of the By-Laws changed so that "honorary membership" becomes "award of honor." An award of honor may be bestowed by the Association upon any person who has been a member of the Association for not less than 25 years and who has made a distinguished contribution to the Association.

(5) Article III, Section II of the By-Laws changed so that Life Membership may be granted

(a) To active members who retire after having belonged to the Association for 25 years or more.

(b) To an active member who has paid membership dues, to include those of a student affiliate, for a least 40 years.

(6) Changes have been made in the Administrative Handbook by including: (a) the new nominating committee procedure; (b) the Paul Lewis Award rules; (c) the membership committee procedure.

(7) The following have been elected to Life Membership: J. C. Knott, Pullman, Washington; F. H. Herzer, State College, Mississippi; C. J. Schneider, Grand Ledge, Michigan; W. M. Regan, Davis, California; J. V. Quigley, Kansas City, Missouri; T. A. Baker, Newark, Delaware; and J. L. Hileman, Syracuse, New York.

(8) Approved the establishment of Student Affiliate Chapters at the University of Maine and South Dakota State College.

(9) Approved $100 cash award and plaque from Association funds for the most outstanding student chapter for 1959. Also approved $100 cash award from Association funds for winner of scientific paper presentation.

(10) The following officers were elected for the year 1959-60: A. C. Fay, President; R. E. Hodgson, Vice-President; G. H. Wise and F. J. Doan, Directors.

III. *Election of life members.* The following were elected to Life Membership: T. W. Gullickson, University of Minnesota; M. H. Campbell, University of Rhode Island; and P. S. Lucas, Michigan State University.

IV. *Authorization of an assistant to the Treasurer to sign checks.* Voted that Susan S. Hustis, who does secretarial work for Mr. Judkins, be authorized in any emergency to sign checks in payment of Association bills.

V. *Investment of reserve funds.* Voted that the Secretary-Treasurer be authorized to invest up to $20,000 from current reserve funds of the Association in high-yielding, high-grade bonds.

VI. *Scientific article abstracts.* Voted that we give H. F. Judkins and E. O. Herreid a vote of confidence and appreciation for the manner in which they are handling the popular JOURNAL abstracts.

VII. *Method and time of collecting dues and granting membership cards.* Voted that the Executive Board approve the procedure outlined by the Secretary for the handling of dues, notices, membership cards, and dairy science abstracts (English) for 1960.

VIII. *Travelling expenses to committee meetings.* Voted that the Board encourage an interim meeting during the 1959-60 year of the following committees, with the understanding of the committee members that expenses not approved by home institutions or companies will be paid by the Association. The committees are: Policy, Program, Journal Management, Education, and Student Affiliate.

It is further recommended that the President and Secretary be authorized to approve the travel expense for meetings of other committees within the limitations of the travel budget.

IX. *American Institute of Biological Sciences membership.* Voted to discontinue membership of A.D.S.A. in the American Institute of Biological Sciences.

X. *Acceptance of future award offers.* Voted that the Board accept the awards offered by the following companies for 1960: National Dairy Products Corporation, The Borden Company, The DeLaval Separator Company, The American Feed Manufacturers (also 1961 and 1962), and The Paul Lewis Laboratory for the year 1960, and that the Secretary be directed to extend the thanks of the Association and the Board to these companies and Associations for the continued interest in and support of our awards program; at the same time, explaining the competition involved in the selection of the winner.

XI. *Codifying of Board meeting minutes.* Voted that the Secretary be instructed to examine codification of Board Minutes to determine those items in the Minutes which are no longer operating practices of the Association, and that these be referred to the Executive Board either by mail or at the next meeting so that appropriate Board action can be taken to bring the Code in conformity with the current practice. It is further moved that the corrected coded Minutes be made a part of the permanent administrative Handbook.

XII. *Future meeting dates.* Voted that we accept the kind invitation of the University of Georgia to hold the meeting at that institution in the year 1964. The 1960 meeting will be held at Utah State University, June 19 through 22.

XIII. *Publicity for annual meeting.* Voted that the publicity committee, consisting of Messrs. Judkins, Herreid, and Herman explore the best methods for a complete publicity program of the Annual Meeting before, during, and after the meetings, and that a due date of January 1 be set for the report of this committee by letter to the Executive Board.

XIV. *Necrology report.* The passing of the following members has been reported to the Secretary since the 1958 meetings: N. N. Allen, Madison, Wisconsin; C. A. Altwegg, Escondido, California; R. L. Anderes, Kansas City, Missouri; A. E. Banks, Glendale, Arizona; L. S. Bauer, Columbus, Ohio; A. A. Borland, State College, Pennsylvania; P. M. Brandt, Corvallis, Oregon; K. V. Bryan, Lafayette, Indiana; L. R. Bryant, Guelph, Ontario, Canada; E. W. Cheng, Ames, Iowa; W. B. Combs, St. Paul, Minnesota; O. A. Jamison, San Antonio, Texas; W. H. Riddell, Burlington, Vermont; E. L. Willett, East Lansing, Michigan; and G. E. Gordon, Berkeley, California.

XV. *Award nominations study.* Voted that the President appoint a committee to study and develop a plan for preparing dossier material for the several awards.

XVI. *Student branch of A.D.S.A.* Voted approval of the establishment of a student branch of A.D.S.A. in accordance with the Constitution and By-Laws presented by the Student Affiliate Committee of A.D.S.A.

Voted that the Board take steps to amend the By-Laws to permit the amalgamation of all Student Affiliate Chapters into an A.D.S.A. student branch. Such steps to include a ballot to the members with the dues notices in October.

Voted that the student branches be advised that their annual reports are due in the Secretary-Treasurer's office no later than June 1 each year.

XVII. *Expenses of invited speakers.* Voted that the Association defray the registration fees of invited nonmember speakers on the program on request of the host institution, but that the Association shall not be obligated for travel and/or subsistence for invited speakers. In exceptional cases, where outstanding speakers are necessary to the success of the program and expenses must be paid, prior approval to invite speakers shall be obtained from the President and Secretary-Treasurer acting for the Executive Board. It will be the duty of the section program chairman to inform the host institution of the registration arrangements for the guest speaker.

XVIII. *Election of Secretary-Treasurer.* The Board voted to re-elect H. F. Judkins as Secretary-Treasurer of the American Dairy Science Association, with special thanks for his continued devotion to the work of this Association and for the sound, aggressive, and business-like manner in which he has performed the many and varied duties of his office.

XIX. *Election of the Editor.* The Board voted to re-elect E. O. Herreid to the position of Editor-in-Chief of the JOURNAL OF DAIRY SCIENCE for 1960, with a special vote of confidence for the manner in which he is conducting the duties of his office.

XX. *Selection of Journal Management Committee member. The Board* selected J. F. Cavanaugh as a member of the Journal Management Committee, making the committee: J. T. Reid, 1960 Chairman; F. J. Doan, 1961, J. F. Cavanaugh, 1962, E. O. Herreid, Ex Officio, and H. F. Judkins, Ex Officio.

XXI. *Selection of Policy Committee.* The Board appointed the following Policy Com-

mittee: Walter Price, Chairman 1960; Ira Gould, 1960, I. W. Rupel, 1961, L. A. Moore, 1961, Floyd Arnold, 1962, J. Hoffman Erb, 1962, H. F. Judkins, Ex Officio.

XXII. *Selection of Program Committee Chairman for 1959-1960.* The Board selected N. L. Jacobson of Iowa State College.

XXIII. *Appointment of Historian.* The Board appointed G. Malcolm Trout as Historian of the Association.

XXIV. *Resolution re : retiring members of the Board.* Whereas, Elliott Parfitt and George Werner are completing their 3-yr. term of office on the Executive Board and Donald V. Josephson is completing 6 yr. of service on the Executive Board and, whereas, they have served their Association with distinction: Therefore, Be It Resolved that the remaining members of the Board extend their thanks to these colleagues and our regrets that they will no longer be with us.

XXV. *Recognition of absent charter members.* The Board voted the President should write to the absent charter members expressing the good wishes of the Board.

XXVI. *Liaison between Board and sections.* The Board voted that the President should write the vice-chairman of each section to meet with the Board as observers, to develop liaison between the Board and the sections.

XXVII. *Graduate student paper presentation.* After listening to the very favorable comments of the Chairmen of the Student Affiliate Committee and the judges of the 1959 contest, the Board recommended that the contest be continued and expanded to include the Manufacturing Section in 1960, the Student Affiliate Committee to work out the matter of Awards and donors.

XXVIII. *Affiliate chapter award program.* The reports of the judges of this year's Award were very favorable. It was suggested that the exhibits place more stress on the educational value of the work of the Student Chapter. After hearing the judges' report, the Board voted that the Student Affiliate Chapter Award Program be continued and that the prizes awarded be made by the Association, the details being left to the Student Affiliate Committee.

XXIX. *Dairy manufacturing extension group.* The Board voted to recommend that the Manufacturing Section Program Committee give committee status to a member of the Dairy Manufacturing Extension Group, for the purpose of developing a subsection program on practical dairy manufacturing problems at the 1960 meeting.

XXX. *Approval of important section actions by the Board.* Voted that in advance of next year's meeting, the Secretary ask Section Chairmen to submit important questions on which they want Board action, so that they can be discussed and acted upon at Board meetings.

XXXI. Dairy Science Abstracts (English) for 1960. The Board voted to accept the offer of the Commonwealth Agricultural Bureaux for 1960 on the same basis that we have been working for 1959.

XXXII. *Tribute to President Turk.* The Board voted to commend Past President Turk for the expeditious and wise manner with which he has handled the affairs of the Association during the past year and particularly for keeping the Board within bounds.

COMMITTEE REPORTS

The Board took action as follows:

I. *Association Policy Committee.* The Board accepted the report of the Policy Committee, with the following recommended actions on the eight points outlined in the Policy Committee report:

(1) Dairy economics and management section

Approve the idea of encouraging the membership of people working in the fields of dairy economics, dairy marketing and management and their participation in Association activities and programs. Approve the formation of a special committee to study the possible establishment of a Dairy Economics section. It is recommended that the committee request university dairy department heads and others to contact the agriculture economics people in their areas to determine interest and report their findings to the Board.

(2) The Awards ceremony

We see no urgent need for changing our present policy of awards presentations.

(3) A distinguished service award

Recommend the approval of the idea of initiating the A.D.S.A. Distinguished Service Award, with the stipulation that it be limited to one or more (rather than one to five) and that the recipients be limited to those engaged in commercial work, public welfare, and regulatory activities relating to the dairy industry and who may or may not be members of this Association. A committee shall be appointed by the President in accordance with the procedure outlined in the report. This committee will report back to the Board at the 1960 annual meeting.

(4) Achievement award for graduate study

In view of the similar objectives outlined for the Graduate Achievement Award and our present (experimental) Scientific Paper Presentation Award, it is the recommendation of the Board that we await the results of our present program before considering the adoption of another or additional graduate student award plan. It is suggested that the present pilot program might be converted into a combined plan supported by outside Award funds.

(5) Young men of the year

While the Board agrees in principle with the idea of developing leadership from among the younger members of the Association, it is our judgment that the suggested method of implementation would be burdensome, frequently embarrassing to the member or to the Association, and might complicate the affairs of the Sections. It is felt that our objectives can be accomplished by careful selection of appointees to committees and other assignments.

(6) Deadline for presentation of abstracts of papers for the annual meeting

The Board moved to accept the report that deals with the preparation and handling of abstracts of papers presented at the Annual Meeting. It is recommended that the report be referred to the Journal Management Committee for immediate study and for recommendations back to the Board by September 1, 1959.

(7) Association reorganization

The Board feels that the present administrative organization is adequate to implement our present scope of program and that the suggested reorganization seems unnecesary under present conditions.

(8) A popular journal

The Board moved to accept the recommendation of the Policy Committee concerning the possibility of publishing a new popular journal or expanding the popular section of our present Journal, and which suggests that the Executive Board initiate a further study of the problem and its implications. The Board directs the President to appoint a committee of three, plus the Secretary-Treasurer and Editor-in-Chief as Ex Officio members, to study the matter thoroughly and report back to the Board at the next annual meeting.

The Board moved that the Policy Committee be especially commended for its fine work of the past year.

II. *Journal Management Committee.* The Board approved the report of the Journal Management Committee.

The committee recommended that titles and abstracts of papers for the annual meeting be submitted to the Chairman of the Program Committee by March 1. It was further recommended that titles and abstracts be published, officially, in the June instead of the May JOURNAL. It was assumed that the host institution would publish the program the same as had been done in past years.

It was recommended that the call for papers for the annual meeting appear in the September instead of the November JOURNAL, and that a reminder of this call appear in succeeding JOURNALS through February.

III. *Report of the Editor.* The Board accepted the report of the Editor-in-Chief, with special commendation for his continued efforts to improve the scope and quality of the JOURNAL OF DAIRY SCIENCE.

IV. *Report of the Secretary-Treasurer.* The Board voted to accept the report of the Secretary-Treasurer, the important items of which are as follows:

FINANCIAL

Income	$84,561.52
Expenses	80,289.79
Gain	4,271.73

STATISTICS ON MEMBERS AND SUBSCRIBERS AS OF JUNE 12, 1959

(1) Number of 1959 members — 2,558, of which 2,180 are renewals and 378 are new. This compares with 2,457 members for the entire year 1958.

(2) Number of 1958 members who failed to renew—296, as against 378 new members obtained.

(3) Number of 1959 members deceased—15.

(4) Number of 1959 Student Affiliate members—691, as compared to 617 for 1958. The rather marked increase in total number has come from the four-year college classes.

(5) Of the 691 Affiliate Members for 1959, 289 are renewals and 402 are new ones.

(6) Number of 1959 JOURNAL OF DAIRY SCIENCE subscribers is 1,547.

(7) Total circulation of the JOURNAL OF DAIRY SCIENCE for May, 1959, is 4,830.

The Board voted:

(1) That advertising rates be increased by 20%, effective at the expiration of present contracts.

(2) That a plan be worked out between the Secretary-Treasurer and the Editor to bring certain papers to the attention of selected industry representatives and associations, with the suggestion that they purchase and distribute reprints to their customers or members.

(3) That, at appropriate intervals, consideration be given to including a special sheet in the JOURNAL to (a) promote response to advertising; (b) facilitate the ordering of reprints, and (c) facilitate the ordering of the new Plant Training Manual.

V. *Budget for 1959–60.* The Board approved a budget for 1960 totalling $94,223.00.

VI. *Auditing.* The Board approved the report of the Auditing Committee, which indicated a balance of $4,271.73 for 1958 and a present net worth of $39,752.55.

VII. *Dairy Industry Plant Training Manual.* The Board moved the acceptance of the report of the Plant Training Manual Committee, with the recommendation that the Secretary-Treas-

urer be authorized to proceed with arrangements with the Garrard Press for printing and distributing the manual in book form. It is recommended that the sale price of the book be set at about $4 postpaid, and that the project be temporarily financed from cash reserves in the Association treasury. It is further recommended that the Committee now be discharged, with a special commendation from the President on behalf of the Board for a job well done.

VIII. *Public Health.* The Board accepted the report and commended the Public Health Committee and especially the four subcommittees that have reported this year for their diligence and for their excellent reports.

We recommend the publication in the JOURNAL OF DAIRY SCIENCE of the reports of the subcommittees on Animal Disease Control, L. A. Black and W. D. Pounden, and on the Foreign Substances in Milk and Milk Products, L. L. Ramsey. We suggest that Mr. Ramsey be requested to condense his report where possible and that he add in the last paragraph on page 12 following the "U.S. Department of Agriculture" the words "especially the Agricultural Extension Service."

Because of recent publications of articles covering essentially the same material as that presented by the subcommittee on Antibiotics, R. B. Parker and W. A. Krienke, and by the subcommittee on Radiation, E. B. Kellogg and W. C. Parkinson, we do not recommend that these reports be published in the JOURNAL OF DAIRY SCIENCE.

IX. *Membership.* The Board accepted the report and commended the Membership Committee and Secretary-Treasurer Judkins for achieving and passing their goal of 2,500. We urge that their recommendations as presented in the report be adopted. The organization, with the cooperation of H. F. Judkins, is a most effective one.

X. *Education.* The Board accepted the report and commended the Education Committee for their continuation of programs of broad interest, and urged that their recommendation for a program period that would not compete with other programs be granted.

XI. *Dairy Remembrance Fund.* The Board expressed their appreciation to the Dairy Remembrance Fund for their support of educational and scientific endeavors in dairy science. We commend the committee on their liaison and for keeping A.D.S.A. informed.

XII. *National Research Council.* The Board accepted the report and commended Dr. W. E. Krauss for long and effective representation of the A.D.S.A. to the National Research Council. The present is an excellent report.

Dr. Krauss is to be congratulated on his position as Chairman of the Agricultural Board of the Division of Biology and Agriculture.

This subcommittee recommends that A.D.S.A. express its support of the position of the Executive Committee of the Division with respect to the breadth of interest with which the Division should concern itself.

The Board approves the publication in the JOURNAL of the report of the representative to the National Research Council after it is reedited by the author.

XIII. *Resolutions Committee.* The report of the Resolutions Committee was approved.

Report of the American Dairy Science Association Resolutions Committee

Your Resolutions Committee, after considering the various suggestions made by committees and sections of American Dairy Science Association, plus those received from the heads of dairy departments of colleges and universities, wishes to submit the following resolutions which have been approved by the Board of Directors:

RESOLVED, that the American Dairy Science Association extend its sincerest appreciation to the staff of the University of Illinois and to the personnel of other agencies in Illinois for contributing so much toward the success of this the 54th annual meeting of the Association. Our special thanks go to the members of the departments concerned, and their families, for the fine hospitality they have shown us during our stay here.

RESOLVED, that the American Dairy Science Association extend its appreciation to the Borden Company, the American Feed Manufacturers' Association, the DeLaval Separator Company, to the Milk Industry Foundation, the National Dairy Products Corporation, and The Paul Lewis Laboratories for the generous awards given annually to members of this Association, in recognition for outstanding work performed in the fields of research, college teaching, and extension. These are wonderful examples of industry cooperating with those engaged in research and educational endeavors, in an effort to increase the contributions of dairy science to the welfare of mankind.

WHEREAS, the American Dairy Science Association is holding its annual meeting on the campus where this Association was founded in 1906 under the leadership of Professor Wilbur J. Fraser, therefore it is

RESOLVED, that we express our continued appreciation for his farsighted action; and it is further

RESOLVED, that a copy of this resolution be sent to Mrs. W. J. Fraser.

WHEREAS, a review of the literature on the subject reveals how limited our knowledge is on the biological effects of low-level radionuclides, therefore it is

RESOLVED, that the American Dairy Science Association support efforts to continue and expand intensive research in the four fields listed below:

(1) Coordinated studies on the occurrence of strontium^{90} in all basic components of the diet.

(2) Development of methods of preventing or limiting strontium^{90} from entering the food supplies of animals and man.

(3) Investigation of techniques for the removal of strontium^{90} and other radionuclides in foods.

(4) Development of basic information on the mode of action of low levels of radionuclides, including studies to determine if the relationship between dose and effect is linear at all levels of exposure, or if there is a threshold of exposure below which there is no effect; and

WHEREAS, the projects outlined above are beyond the means of any one interested group, therefore it is

RESOLVED, that the American Dairy Science Association urges that federal and state governments, and the food and related industries, assist in providing the leadership and funds necessary to develop the facts needed in the best interests of public health.

WHEREAS, there is a critical need for a strong educational program on the proper use of pesticides, herbicides, antibiotics, drugs, and food additives, in order to assure a continued safe and pure supply of milk and milk products, therefore it is

RESOLVED, that the American Dairy Science Association through its committee on public health join forces in a comprehensive educational program with the Food and Drug Administration, the U. S. Department of Agriculture, the Agricultural Colleges and Extension Services, the U. S. Public Health Service, state and local regulatory agencies, national farm organizations, and national industry organizations.

WHEREAS, the bacteriological examination is an integral part of the sanitary and quality control of dairy products, and

WHEREAS, there has been formed an ad hoc committee of interested bacteriologists representing various professional societies to establish an independent certifying commission which will be charged with the responsibility of insuring constant growth-promoting qualities of certain bacteriological media which may be manufactured by any media manufacturer, therefore it is

RESOLVED, that the American Dairy Science Association endorse the principle involved in the certification of bacteriological media to insure constant growth-promoting qualities of these media and, furthermore, it is

RESOLVED, that the American Dairy Science Association encourage the formation of an independent certifying commission consisting of professionally qualified scientists, who will recommend constant growth-promoting bacteriological media for the examination of milk products.

RESOLVED, that the American Dairy Science Association again strongly urge that increased support be given to the Dairy Husbandry Research Branch of the USDA, in aiding the production-testing and sire-proving programs under way in this nation.

Respectfully submitted,

C. H. PARSONS
B. L. HERRINGTON
R. B. MAXY
J. T. MILES
R. E. LEIGHTON

XIV. *Historian.* The Board accepted the report and commended Dr. G. M. Trout for his work as Historian, and particularly for the recent codification of the affairs of the Association. We also commend Secretary Judkins for indexing the Code and putting it in a more useful form.

XV. *3-A Standards.* The Board accepted the report and commended this committee for representation of A.D.S.A. to the 3A Standards Committee and for keeping the Association informed. We support the suggestion for continuity of personnel on this Committee. We recommend that all of these committees (see typed assignment) be continued without change of assignment.

XVI. *Pasture and range management.* The Board accepted the report and commended this committee for their activities in assisting with the revision of the report "Pasture and Range Research Techniques." We recommend the Committee be continued until the completion of this task.

XVII. *Quality standards for production of manufacturing milk.* The Board recommends the acceptance of the report and thanked the committee for their participation. Inasmuch as the work of the committee apparently is completed, the committee is discharged.

XVIII. *Program Committee.* The Board accepted the Program Committee report with commendation.

The Board feels they should not initiate action on dates of the month of June or the days of the week for holding the A.D.S.A. meeting but that, rather, these should be decided at the convenience of the host institution and approved by the Board. Since much of the success of the program is due to thorough prior planning of the Program Committee, the Board recommends continued use of a Program Time Table. The portion of the Program Time Table of interest to A.D.S.A. members should be considered by the Journal Management Committee for possible publication in the JOURNAL, to facilitate total program planning. If this information is to be published, the General Program Chairman should prepare an appropriate article for the JOURNAL.

XIX. *Student Affiliate Committee report.* The Board recommends approval of the Student Affiliate Committee reports. The Board commends the work of the committee members and appreciates the cooperation of Secretary Judkins, who assisted in accomplishing an 18% increase in Student Affiliate members this past year. The Board anticipates receiving the report for next year pertaining to the establishment of a Student Branch of A.D.S.A.

XX. *Dairy-wide committee on nutrition research.* The Board approved the report of the A.D.S.A. representative to the Dairy-wide Coordinating Committee on Nutrition Research. The Board commends the work of Dr. K. G. Weckel for his excellent participation on this committee. Since the work of the Nutrition Research Committee has been discontinued, the Board need not appoint a representative to the Dairy-wide Coordinating Committee on Nutrition Research.

XXI. *American Association for the Advancement of Science.* The Board approved the report of the representative of A.D.S.A. to A.A.A.S. The Board recommends continued representation to A.A.A.S. Furthermore, the Board commends Dr. R. E. Hodgson for leadership in initiating the program "Germ Plasm Resources in Agriculture: Development and Protection" to be held December 19, 1959. If appropriate, the Board recommends to the Journal Management Committee that the papers of the program relevant to the dairy industry be published in the JOURNAL OF DAIRY SCIENCE.

XXII. *Purina fellowships.* The Board congratulates the committee on the men selected to receive this fellowship. The Board commends the Ralston Purina Company for their financial support in the encouragement of capable students to continue their education for the furtherance of themselves and their contribution to the dairy industry. The Board appreciates the efforts of Professor J. B. Frye in selection of the best possible recipients to represent the dairy industry.

XXIII. *U. S. Livestock Sanitary Association.* The Board approved the report of the A.D.S.A. representative to the United States Livestock Sanitary Association. The Board commends the action of the Legislative Committee of the United States Livestock Sanitary Association in seeking increased funds for the detection and control of disease. Research and education concerning tuberculosis in cattle should receive special consideration. The Board recommends that this excellent report by Professor C. G. Bradt be summarized for the portions relevant to cattle, and that it be published in the JOURNAL OF DAIRY SCIENCE.

XXIV. *National Association of Artificial Breeders.* The Board approved the report of the representative of A.D.S.A. to N.A.A.B. The Board encourages continued active financial support of N.A.A.B. for research in dairy cattle breeding. The Board recommends that the Production Section and the Extension Section consider taking action on the resolutions proposed for adoption by N.A.A.B. at their Eleventh Annual Convention.

XXV. *American Institute of Biological Science.* The Board accepted the report of the A.D.S.A. representative to the American Institute of Biological Science. Mr. D. L. Hill is to be commended for his participation on the committee. The Board recommends that the A.D.S.A. not continue as an affiliate member of American Institute of Biological Sciences.

XXVI. *Inter-society cooperation.* The Board approved the report of the A.D.S.A. representative to the Policy Committee of Scientific Agricultural Societies. The Board recommends that A.D.S.A. no longer participate in the activities of the Policy Committee of Scientific Agricultural Societies. Furthermore, the Board requests the President of A.D.S.A. to write to Dr. W. H. Gorman of the Policy Committee, 1700 K Street, N. W., Washington, D.C., and notify him of the Board's action. The Board commends Dr. L. A. Moore for his excellent work on this committee.

XXVII. *Dairy Industry Training Committee.* The Board approved the report of the A.D.S.A. liaison representative to the Subcommittee on Education and Training of the Dairy Industry Committee. Since this subcommittee has been inactive this past year, the Board recommends that an A.D.S.A. representative not be appointed for 1959-50. Furthermore, the Board expresses sincere appreciation for the interest and work of Professor H. B. Henderson in serving as the A.D.S.A. representative.

XXVIII. *International Dairy Congress.* It was voted that the Board approve the report of the special committee on the International Dairy Science Congress. This report recommends the cancellation of plans for an International Dairy Science Congress in 1962, because of the plans already formalized for a meeting in Denmark in 1962. It was further voted that the committee be discharged, with thanks for a job well done. It was voted that the Board appoint a special representative of the Association to coordinate the activities between A.D.S.A., USDA, the Land Grant College Association, the University of Maryland, industry groups, and others who may be cooperating in the USDA and Land Grant College Centennial features of the 1962 meeting.

In view of the fact that preliminary plans for holding the International Dairy Congress in the United States in 1962 have been cancelled in favor of the prior invitation from Denmark, it is recommended by the Board of the American Dairy Science Association that preliminary steps be taken to obtain the necessary support for a similar Congress to be held in the United States in 1965, and that Dr. Ralph Hodgson and other members of the A.D.S.A. attending the 1959 International Dairy Congress be authorized to explore the possibilities for holding such a Congress in the United States.

REGISTRATION DATA

Total number registered

Men	
Members	762
Affiliate	190
Nonmembers	100
Others	4
	1,056
Women	254
Children	222
Total	1,532
Number of men registering from universities	751
Other male registrants	305

Eighty per cent of those attending preregistered.

The following foreign countries were represented: Australia, Canada, Chile, Colombia, England, Greece, Japan, Philippines, and Sweden.

REPORT OF THE MANUFACTURING SECTION

The manufacturing section held two business meetings on Monday, June 15, 1959, Chairman B. H. Webb presiding. The following officers were designated by election to serve for 1959-60: S. Patton, Chairman (Pennsylvania); B. Heinemann, Vice-Chairman (Missouri); and M. L. Speck, Secretary (North Carolina).

The report of the Nominating Committee was presented by S. T. Coulter. J. M. Jensen presented a report on the 3-A Sanitary Standards Committee.

B. L. Herrington reported for the Resolutions Committee. At his request, M. L. Speck reviewed the need for certification of bacteriological media. He moved a resolution be adopted that the American Dairy Science Association endorse the principle of certifying bacteriological media to insure that they have constant growth-promoting qualities, and that the American Dairy Science Association encourage the appointment of an independent certifying commission of qualified scientists, who would recommend media of constant growth-promoting qualities for the examination of milk products. The motion was passed unanimously.

G. M. Trout reported on the very active committee of Judging Dairy Products which published four papers in the JOURNAL OF DAIRY SCIENCE during the past year. This report was accepted and the committee was continued.

C. Harper reported for the Committee on Butter and recommended that a final draft for the definition of butter be drawn up to be presented at the next annual meeting. It was also suggested that the recommendations of the 1958 committee be activated. The report of the committee was accepted and the committee was continued.

L. W. Aurand reported for the Committee on Curd Tension. The Committee recommended that the Cherry-Burrell Curd Meter be approved for use in the Official Curd Tension Method. The committee reported wide interlaboratory variations in results obtained on identical samples. It was, therefore, recommended that the committee be discontinued until such a time as basic work had been done on the method. The report of the committee was accepted.

J. R. Brunner reported for the Committee on Milk Protein Nomenclature, stating that there was no absolute agreement among the members of the committee concerning methods of measuring the boundary in electrophoretic measurements. The Committee also recommended continuation of the policy previously adopted by this section, relative to the scheduling of two or three invited papers as part of the milk protein symposium. The report of the Committee was accepted and the Committee continued.

B. M. Zakariasen reported for the Committee on the Development of a Score-Card for Dry Milk Products. Such a score-card was prepared and accepted by the section. The Committee considered its work completed and was discharged.

It was moved, seconded, and passed that a new committee be appointed to devise a score-card for sterile and concentrated milks.

Respectfully submitted,
BYRON H. WEBB, Chairman
STUART PATTON, Vice-Chairman
B. HEINEMANN, Secretary

REPORT OF THE PRODUCTION SECTION

The Production Section business meeting was held June 15, with Chairman N. L. Jacobson presiding. Minutes of the preceding meeting were read and approved.

Officers elected for 1959-60 were: Chairman, G. W. Trimberger (New York); Vice-chairman, S. D. Musgrave (Oklahoma); and Secretary, J. C. Thompson (Missouri).

The Dairy Cattle Judging Committee reported a need in method of presentation of awards at the National Contest. A means of recognizing individual, team, and breed awards was presented. The Committee recommended that discussion and action on the request of the American Milking Shorthorn Society, to be included in the 1959 and subsequent contests, be delayed until the coaches' meeting prior to the 1959 Waterloo contest. The report was adopted as presented.

The report of the Resolutions Committee, expressing appreciation to the Section officers and to the staff members of the Dairy Science Department of the University of Illinois, for

respective contributions to the success of the program, was adopted.

A joint meeting of the Production and Extension Sections was held at 5 P.M., June 15. The report of the Breeds Relations Committee was presented and adopted. The recommendation was made that the responsibilities of the committee be reviewed and revised. This was formalized in a motion by Becker that the Section officers of the Production and Extension Sections review the objectives and responsibilities of this committee and that any action taken should be channeled through the Executive Board. The motion was seconded and carried.

The report of the Dairy Cattle Breeding Committee was adopted as presented. It was recommended that the solids-not-fat special committee continue its work. Combination of natural and artificial service daughters' production records into a single proof was not condoned. The number of herds involved in the proof of a sire should be published by the Agricultural Research Service, USDA. Also, if the only record of a cow was less than 305 days, because of abortion, then it should be extended and used in the sire proof.

The report of the Type Committee was adopted as presented. The recommendation was made that the maximum age of show bulls be the 2-yr.-old class.

The report of the Dairy Cattle Health Committee was adopted as presented. It was recommended that antibiotics not be used in the ration of the milking cow for purposes of disease control or stimulation of milk production. Minutes of a Joint Committee on Dairy Cattle Health of A.D.S.A. and A.V.M.A. were appended.

It was moved by Reaves, seconded, and carried that the officers of the Production and Extension Sections serve as a committee to review the responsibilities of all standing committees of the Production and Extension Sections and recommend appropriate action to the Board of Directors.

Respectfully submitted,
N. L. JACOBSON, Chairman
G. W. TRIMBERGER, Vice-Chairman
S. D. MUSGRAVE, Secretary

RESOLUTIONS REPORT FOR THE PRODUCTION SECTION OF THE A.D.S.A., 1959

(1) RESOLVED, that in recognition of the splendid work of our section officers, Chairman Jacobson, Vice-Chairman Trimberger, and Secretary Musgrave, who have organized a well-balanced and informative program for our 54th annual meeting; we, the members of the Production Section, express our sincere appreciation for a job well done. In addition, we recognize that much of the work of the Production Section is done by its committees and take this opportunity to express our appreciation to the members of these committees for the fine work they have done.

(2) RESOLVED, that the Production Section of A.D.S.A. extend its sincerest appreciation to the staff of the University of Illinois and to the personnel of other agencies in Illinois for contributing so much toward the success of this 54th annual meeting of the Association. Our special thanks go to the members of the Department of Dairy Science and their families for the fine hospitality they have shown us during our stay here.

Respectfully submitted by:
THE RESOLUTIONS COMMITTEE
C. L. NORTON
R. E. MATHER
J. T. MILES, Chairman

REPORT OF THE EXTENSION SECTION

The business meeting was held June 15, 1959, with Chairman L. A. Johnson presiding. Officers elected for 1959-60 were: Chairman, C. W. Nibler (Nebraska); Vice-Chairman, Marvin E. Senger (North Carolina); and Secretary, W. R. Van Sant (Arizona). Tribute was expressed on behalf of the passing of fellow extension workers, Frank Fitch, Leonard Trunnel, and G. E. Gordon, during the past year.

The following committee reports were presented and accepted:

Dairy records. A record program to be known as Dairy Herd Improvement Registry (DHIR) was approved. This program was developed through the joint efforts of the Records Committee, the Breed Relations Committee, and the PDCA Testing Committee. It provides for the acceptance of machine-processed DHIA records by Breed Registry Associations for use in their breed improvement programs similar to HIR records. Uniform herd-book covers for DHIA, owner-sampler, and WADAM records were adopted. Uniform lactation and yearly herd reports were recommended for reporting data to Dairy Herd Improvement Section, ARS, USDA, by dairy record processing centers. A national testing year for summarizing data was recommended.

A demonstration and report of studies made on the TeSa butterfat test were given. Following previous action of this Section, the committee recommended that all data be presented to the Association of Official Agricultural Chemists for further evaluation and specific approval of this test for use in production testing. It is recommended that greater emphasis be given to field checking of automatic weighing and sampling devices now in use.

Greater uniformity in the use of a standard ear-tag for cow identification in DHIA, artificial breeding, and disease control is being encouraged. A system of sealed neck-chains or straps is being developed for more practical identification by testers within herds.

Several changes in the rules were recommended in keeping with the late developments in the testing program. A suggested representation for this committee was given. An amendment was passed to add the chairman of the PDCA Testing Committee as a member of the Dairy Records Committee.

4-H Clubs. Establishment of a score-card for use in judging dairy heifers was recommended. Also, that more attention and training be given the group system of judging often used at 4-H shows at PDCA-sponsored judging schools. Recommendations were given pertaining to premium schedules for use in 4-H dairy shows. Papers were recommended and presented on the feeding and management of dairy calves and heifers.

Teaching methods. Eleven states presented exhibits on effective ways and methods of extension teaching used in their respective programs. A paper was presented on good, bad, and indifferent visuals for extension teaching.

Resolutions. No resolutions were presented by this committee.

New business consisted of a proposal to dissolve the Breed Relations Committee, a joint committee with the Production Section. It was moved and passed that the officers of the Extension and Production Sections serve as a committee or appoint a committee to study the duties and responsibilities of all joint committees and eliminate or reassign duties as may be appropriate. This motion was accepted in the joint business meeting, amending it to include all standing committees.

Twelve papers were presented in the general areas of production testing, feeding levels as related to growth and production and automation in dairy cattle feeding and milking. Abstracts of papers have been published and copies have been distributed to all extension dairymen.

Respectfully submitted,

L. A. JOHNSON, Chairman
C. W. NIBLER, Vice-Chairman
MARVIN SENGER, Secretary

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Frequent inquiries are received for prices of reprints from organizations in the dairy industry and from those serving the industry in technology and in production. For this reason it was decided to publish the latest American Dairy Science Association price schedule for reprints of papers that appear in the Journal of Dairy Science. It is hoped that this price schedule will make it easier for interested people to obtain reprints in any number desired and at the same time aid in disseminating useful information to the entire dairy industry and to its related industries. The schedule is as follows:

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THE SURFACE TENSION OF MILK. A REVIEW[1,2]

C. H. WHITNAH
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SUMMARY

Methods of measurement of surface tension applicable to milk have been reviewed. Objectives of surface tension studies included: detection of adulteration; relations of surface tension to the formation of foams or emulsions; to bacterial growth; to the species, ass, buffalo, cow, goat, mare, whale, and woman; to heat treatments; to homogenization; and to developed off-flavors. Milk derivatives studied include: (a) solutions of the proteins casein, β-lactoglobulin, Rowland albumin and globulin, and σ-proteose; (b) wheys, acid, rennet, salt, or ultrafilter; (c) milk acidified with hydrochloric, lactic, or butyric acid; and (d) cream, ice cream, or evaporated milk. Free fatty acids and proteins appear to be the surface-active constituents causing the surface tension of milk or milk products to be lower than that of water.

Brief reviews in 1936 (8), in 1946 (3), and in 1958 (71) have noted a scarcity of data on the surface tension of milk. A cursory survey of the complexities and uncertainties found in surfaces on surface-active materials in pure liquids, and in mixtures containing only two components, might leave one surprised at the abundance rather than the scarcity of studies on such a complex material as milk. Relationships between a solvent and a single surface-active solute have been extensively studied for application to synthetic detergents. Features that might be applied to milk have been reviewed by Moilliet and Collie (49), who also cite many other reviews. Milk fat, some of the milk proteins, and free fatty acids are some surface-active constituents of milk which undoubtedly affect both surface properties (e.g., surface tension) and also bulk properties (e.g., micelle or globule formation) in milk or liquid milk products. Clayton (14) has used milk and modified milk as illustrative material for a general study of many problems in surface activity. Rules have been suggested for relationships between the surface tension and vapor pressure of mixtures (79). These rules have also been applied to other physical properties (e.g., compressibility). It was found they do not always hold, even for binary mixtures (15).

METHODS OF MEASUREMENT

Methods used in measuring the surface tension of milk have been described as static, dynamic, or semidynamic. A static method should yield an equilibrium value, or at least a value that does not change during several times the interval required for measuring. Careful studies on the static surface tension of milk have used capillary rise, the DuNuoy ring, the Wilhelmy plate, or bubble pressure. Methods, using the weight of a falling drop or the number of drops falling from a given volume of milk, have been called dynamic by some workers and semi-

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[1] Contribution No. 585, Kansas Agricultural Experiment Station.

[2] Reprints available for 90 days after publication of this paper. For prices refer to page 1434 of the August issue of this JOURNAL.

dynamic by others. The shape of a pendant drop may be described as a dynamic method when the time of changing surface composition is long compared to the time of formation of the drop. However, the same uncertainty as in the falling-drop methods arises when the time of measurement is near the time required for formation of the drop. The pendant-drop method apparently has not been used for milk or milk products. The name semidynamic has been used to describe a value which includes to an uncertain degree both the age of the surface and the approach to surface equilibrium. DuNuoy or Wilhelmy methods applied to the surface on a continuously overflowing container have also been called dynamic (10, 11, 14, 17, 30, 31, 33, 34, 35, 49, 51, 62, 64, 71, 73). A critical bibliographical study of the methods of measurement of dynamic surface tensions has recently appeared (19). Drop numbers for aliquots of a sample of skimmilk, when flow times from a stalagmometer were adjusted by changing the air pressure above the sample, decreased from 59.5 at 21 sec. to 52.8 at 374 sec. (46).

Two recent studies of the vibrating jet method have evaluated and refined its use with jets of surface-active solutions (30) and with jets whose surfaces are retarded by friction (31). The method without these refinements has been used in two studies of milk. Both studies agree that the surface tension of milk did not change during the observed age interval of the jet. The first study concluded that the change had not yet begun and that the surface tension of the jet was equal to that of water (42). The second study concluded that the surface tension of the jet was equal to the static value for milk, or that the change was completed during the early part of the first wave (77). Extension of Bohr's method to a fifth approximation in a jet, where the axial ratio $a/b = 2$, changed the calculated surface tension by 0.8%. A table, expressing values of Bohr's ratio n/m in terms of axis ratio a/b, reduced the time needed to calculate a surface tension value by about half. A telephoto camera lens provided a magnified image while the lens was positioned, so that the jet pictures occupied one quadrant of the lens field (75).

A possible explanation of the above conflict is that the earlier workers did not consider differences in density and viscosity between milk and water. The difference in density probably is not significant. The importance of viscosity differences depends on the average diameters and velocities of the jets. These values were not reported in the earlier work. The large decreases of surface tension, in the later study during the interval 0.001 to 0.01 sec. for diluted milk, or for milk from which proteins had been precipitated, seem to justify the second conclusion.

Capillary rise applied first on filter paper, and later much more exactly in glass tubes, has been rated as the most accurate method for measuring static surface tension (73). Obtaining a satisfactory contact angle for a surface-active solution in a capillary tube has been challenged (51, 62). The capillary rise method also has been vigorously defended under particular conditions for milk products (35, 43, 64). Capillary rise values have been found parallel to, but lower than, falling-drop values (46). They have also been found to "compare

with'' (equal?) values using a Wilhelmy plate method or falling drop methods if the drops formed slowly enough (78).

ADULTERATION

Perhaps the simplest objective in studying the surface tension of milk has been to detect adulteration with water or starch (28, 44). Studies with other objectives have observed effects of added water (3, 77) or ionic solutions (43, 56, 64), and of foreign fats (21) or fatty acids (46, 52). Other features of change in surface tension (3, 46, 77) clearly rule out surface tension as a simple test for adulteration.

FOAMING TENDENCY

Milk is one of several systems where tendency to foam and foam stability have been studied in relation to surface tension (49). Falling-drop numbers were early used to measure surface tension and viscosity in relation to the cleansing foam of soap solutions (33). It has been claimed that ". . . it is not low surface tension per se that ensures foam stability but the nature of the adsorbed layer giving rise to the lowering of surface tension.'' (54). The proteinaceous material eluted by dilute sodium chloride from commercial caseins of high foaming tendency carried the foaming tendency. Small changes in the surface tension of the eluate were found on aging to 6 hr., on boiling, on concentrating at 50° C. and rediluting, and on diluting fivefold. Dilutions of 100- and 1,000-fold produced larger rises in surface tension, and 5,000-fold produced a value slightly higher than water (1). Increased foam stability of aqueous solutions of purified milk proteins with higher concentrations of caseinate, albumin, or lactoglobulin could not be explained by changes in surface tension. The surface tension values were not depressed by concentrations of 0.0001%, but reached minimum values in the range 0.01% to 0.05% (26). The foaming tendency of milk serum, prepared by ultrafiltration of skimmilk, was reduced half by adding 1% skimmilk, without significant change in surface tension (42). At 25° C. or lower, where a phospholipid-protein type foam predominated in simplified systems containing milk constituents, large changes in foaming tendency accompanied only minor changes in surface tension. In some mixtures higher temperatures produced a decrease of 25% or more in surface tension with or without large changes in foaming tendency (60).

EMULSIONS

Emulsion stability and small fat globules are promoted by lowered interfacial tension. The nearly equal lowering of 12.06 and 12.04 dynes/cm for ''hard fat'' and cocoa fat did not explain differences found in emulsifying power. The lowering of interfacial tension also was very uniform for different skimmilks (47). The surface and interfacial tensions between skimmilk and different fat fractions did not differ sufficiently to be of importance in the theory of butter formation (48). Ice cream mixes containing several emulsifiers and/or stabilizers did not have important differences in surface tension. Stabilizers produced small increases, and emulsifiers slight decreases. Averages were: control 50.02, with stabilizer 53.48, with both stabilizer and emulsifier 48.20 dynes/cm (59).

BACTERIAL GROWTH

Bacterial growth as affected by the surface tension of milk should be considered in relation to older studies on effects of surface tension in other media. In media whose surface tension could be depressed to 32 dynes/cm by castor oil soap, it was found that all pellicle formers ceased to grow at surface tensions less than 45 dynes/cm, and that some anaerobes grew aerobically on media of low surface tension (41). The nature of the depressant, the kind of bacteria, and sometimes the salt concentration of the medium were found to be important factors affecting the relation of growth to surface tension (4). Rancidity development in cold milk accompanied a decrease in surface tension. This decrease has been considered the cause of retarded growth of *Streptococcus lactis* in rancid milk (66). Surface tension depressed with Tween 40 did not retard growth of *S. lactis* in milk. Toxic specific fatty acids, rather than lowering surface tension, were suggested as the cause of the above effect. Oleic acid lowered surface tension but not growth rate, whereas caprylic acid lowered growth rate but not surface tension (16).

SPECIES

Surface tension values for milks of seven species are collected in Table 1. Only those values for cow's milk which were measured along with milk of one

TABLE 1
Surface tension of milks from different species

Ref. No.	& Yr.	Species: Ass	Buffalo	Cow	Goat	Mare	Whale	Woman
		(surface tension, dynes/cm)						
40	1911			36.2		37.4		37.4
9	1913		44.0	44.1		44.0		
9	Boiled		46.0	48.4		44.2		
55	1916	56.8		55.2	51.8			50.4
55	Cooled 1 hr. 5° C.	56.2		51.5	50.2			47.4
55	Whey (HCl)	51.5		56.0	57.0			
55	Whey-cooled	51.2		55.8	56.0			
7	1922			46.7				46.1
7	Shaken			58.6				60.4
7	Skimmed			48.2				48.1
36	1941			47.7	48.6			48.5
53	1953						39.1	
5	1953				47.5			
45	1954			48.1	47.5			
13	1955				48.2			

or more other species are included in this table. Some values have been converted from other units to dynes/cm, and some averages have been calculated from individual reported values. The variations between species in lines do not appear larger than the variations in columns, due either to different observers or to known differences in the treatment of the milk. Treatment with ultrasonic vibrations has been reported to lower the surface tension of women's milk but not the milk of cows or goats (36). Increased casein content was found to de-

crease surface tension, whether the casein particles were large, as in mare's milk, or small, as in women's milk (40).

DERIVATIVES

It was early shown that the surface tension of milk increased with decreased concentration of fat or casein caused, respectively, by skimming or acid precipitation. Replacement of the constituent again lowered the surface tension. Effects of udder inflammations, pregnancy, stage of lactation, and feed also were noted (38). Effects of shaking, heating in various ways, and standing or aging of the solution on the surface tension of solutions of casein in water also have been noted (9). Differences in the relation of the surface tension of whole milk to its acid whey, and of each of these to cooling, have been mentioned (Table 1) for milk of the ass, cow, and goat (55).

Acid numbers in the range 13 to 27 had very little effect on the surface tension of skimmilk, although smaller increases of acidity from added HCl or from added or cultured lactic acid increased the surface tension. Surface tensions at several ages after adding rennet to raw and pasteurized skimmilk are shown in Table 2. The return of the surface tension of the whey to the original value of the skimmilk indicates that caseinate phosphate complex is not the material affecting surface tension. Addition of 0.05% of 3% formalin to milk did not change the surface tension either immediately or after 24 hr. (46).

The surface tension of cream at 3° C. decreased with increasing per cent fat and usually with age. Both surface tension and viscosity were involved in whipping properties (18). One liter of water shaken three days with 1 g. casein and a small amount of toluene dissolved 0.021 to 0.024 g. This solution was forced up through a capillary tube, the top wiped off with filter paper, and the solution allowed to reach its level. Heights were measured at intervals from 10 sec. to 2 hr., and were found to fit the equation $\gamma = e^{-kt^{1/2}}$, i.e., the plot of $X = \log t$ vs. $Y = \log \gamma$ was a straight line (34). A 1% solution of casein in $N/100$ (pH 2.46) HCl and in $N/250$ (pH 6.8) NaOH was studied for effects of age (2 min. to 43 hr.) on surface tension. Changes in pH caused a sharp maximum in surface tension at pH 4.62 and another smaller maximum at pH 3.4. Minima were at about pH 3.8 and 8.5. Values using H_3PO_4 were consistently about one dyne lower than when using HCl. The salts, $NaNO_3$, NaCl, and Na_2SO_4, produced nearly equal linear depressions of surface tension in the concentration range 0 to $M/256$. From $M/256$ to $M/4$ Na_2SO_4 produced about twice the rise

TABLE 2

Surface tension of raw and pasteurized skimmilk vs. addition of rennet

	Minutes after adding:						
	Before adding	0	10	30	50	70	Whey
	(surface tension, dynes/cm)						
Raw	49.0	46.5	47.5	48.8	48.8		49.0
Pasteurized	48.5	45.1	46.7	47.5	48.5	48.5	48.5

produced either by $NaNO_3$ or by NaCl. Glucose 0 to $M/16$ was without effect (34). Within the concentration ranges of normal milk, high protein concentration caused low surface tension, whereas fat concentration had no effect. The change with temperature in the surface tension of milk was similar to changes for butter fat or fatty acids suspended in water (37). Although raw unhomogenized creams and pasteurized homogenized creams had lower surface tensions than milk of 3.7% fat, the surface tensions of 30 and 35% creams would extrapolate to exceed those for milk at slightly over 4,000 p.s.i. (71).

Purified β-lactoglobulin was selected as a suitable protein for use in studying effects on surface tension of two buffers at three ionic strengths, concentrations of 0.05, 0.10, 0.25, and 0.50% protein, and surface ages of 1 to 60 min. Bulk ages up to 1 wk. did not modify surface tension. Except as t approaches zero, the relation of surface tension (γ) to time or surface age (t) was $\gamma_t = \gamma_{1\,min.}/t_b$. No systematic difference was found between acetates of Na and Mg as buffers, although the differences were often large. The two forms of β-lactoglobulin differed in slope of the plot $X = \log t$ vs. $Y = \log \gamma$ at most pH values, although at pH 5.0 to 5.8 the two lines coincided. The plot $X = \log \log$ concentration of protein added to one of these buffers vs. $Y = \Delta\gamma$ (surface tension of buffer minus surface tension of solution) was a straight line. It was proposed that the time change of surface tension involved three stages: (a) diffusion to and adsorption of a protein monolayer on the surface; (b) denaturation of this monolayer (age 1 to 60 min., the only range observed); and (c) effects of multilayers of normal or denatured protein micelles (64).

The surface tensions of casein, albumin, and globulin (Rowland separation) dissolved in buffers were found nearly equal to water at concentrations of 0.0001%. Casein and albumin yielded definitely lower tensions at 0.0005% and were nearly independent of concentrations above 0.1% at 56.5 and 56.0 dynes/cm, respectively. The drop of 0.01% globulin solution was similar to that for 0.0005% solutions of casein or albumin. The value for globulin at 0.05 to 0.5% appeared stable at about 58 dynes/cm, but fell to 51 dynes/cm in 1.0% solution (26). Effects of emulsions of butter fat containing added phospholipid have been discussed under foaming (60).

The casein- and albumin- (Rowland) fractions of milk were found to be more important than the globulin fraction in effects of dilution, up to 100-fold, on the surface tension of milk. Dilution effects were studied for: (a) whole milk, (b) skimmilk, (c) dialyzed skimmilk, (d) a material called σ-protose, and sera after precipitation of protein with (e) HCl, (f) rennet, (g) $Zn(OH)_2$, or (h) UAc_3. The σ-proteose contained about 3% of the total N of the milk. Either isolated and redissolved, or partially purified in residual milk sera, it was found to account for " . . . the marked surface activity of milk freed from its main proteins" (3). The rates of fall in surface tension with surface age were compared for diluted whole milk, undiluted milk from which the casein had been precipitated by NaCl, and from which whey proteins were also later precipitated by HCl. Results are summarized in Table 3 (77).

When evaporated milk was heated to simulate sterilization in cans, surface

TABLE 3

Rate of fall in surface tension of modified milk

	Diluted $1 \rightarrow 10$	NaCl Ppt. removed	HCl Ppt. removed
Age range sec. $\times 10^4$	20–60–100	10–35–70	10–30–70
Rate of fall dynes/cm/sec	1,700 500	3,200 171	5,500 200

tension increased and provided a better measure of coagulation than visual estimates of graininess. Most of this increase in surface tension occurred during the first third of the heating period, with a further small rise near the end of the heating period. Surface tension values ranged from 50.4 to 58.1 dynes/cm (50).

MILK, WHOLE AND SKIM

A. *Effects of heat treatment.*

Cooling milk for 45 min. or longer to less than 10° C. caused an average decrease in surface tension of 2.12 dynes/cm. An average additional drop of 3.64 dynes/cm occurred on standing 12 hr. Rewarming to 20 or 37° C. caused only a small fraction of the reverse change, whereas rewarming to 50° C. restored the original surface tension value. At 45° C., the restoration was about half complete for milk of 3.8% fat, but less for skimmilk of 0.15% fat. The lowering of surface tension on cooling was also much less for such skimmilk. Skimmilk containing 0.50% fat was very similar to whole milk (6, 12, 67).

Values of surface tension by stalagmometer and by the maximum pull on a straight horizontal wire (stirrup method) for averages of three samples of skimmilk, for averages of four samples of whole milk, and for three cream samples of increasing fat content are shown in Table 4.

Effects of temperature of measurement by stirrup method and of per cent fat were studied on skimmilk mixed with increasing amounts of cream from the same milk. Results are shown in Table 5.

Surface tension at 20° C. (by stirrup method) was measured on two samples of whole milk after six heat treatments. The treatments and values are shown in Table 6.

The surface tension of fresh skimmilk was not changed by storage either at room temperature or at 5° C. (46).

At 18° C., and measured 4 to 6 hr. after milking, DuNuoy values of surface tension ranged from 46.6 to 55.3 dynes/cm, with most samples between 49 and 52. Cooling for 1 hr. at 5° C. reduced tension by 0.9 to 1.5 dynes/cm (69).

TABLE 4

Surface tension relative to method and to fat content of milk

	Skim	Whole	Cream		
Per cent fat	.....	.....	13.5	21.0	36.5
Method	*(surface tension dynes/cm)*				
Stalagmometer	52.7	49.6	46.4	45.5	41.7
Stirrup	46.1	39.5	42.9	42.9	43.1
Difference	6.6	10.1	3.5	2.6	—1.4

TABLE 5

Surface tension vs. per cent fat and temperature

Per cent fat.....	0.04	2–4	10	20	45
Temperature ° C.					
83	38				
20	51.0	46.7	46.2	45.1	44.6
10	51.4	49.0	47.5	47.5	48.5
5	51.6	50.4	49.0	48.7	49.8
1	53				

Surface tension measurements (Wilhelmy at 25° C.) were made on recombined 4% milk to show that the material adsorbed on fat globules differs if the globules are solid or are liquid. The separations were made at 5 or 60° C. with either a hand cream separator or in centrifuged glass tubes. The material was assumed to be adsorbed on solid but not on liquid milk fat globules. For the four combinations containing the material in either the skimmilk or the cream fraction the surface tension of a mixture precooled to 5° C. averaged 2.75 dynes/cm less than for the mixture preheated to 60° C. Corresponding differences with both components enriched or depleted were, respectively, 3.15 and 0.07 dynes/cm (63).

At high temperatures and short times surface tension measurements (DuNuoy at 20° C.) were more sensitive than organoleptic rancidity scores to determine the time-temperature heating needed to inactivate lipolytic enzymes in milk

TABLE 6

Surface tension of whole milk after six heat treatments

Treatment No.[a]	1	2	3	4	5[b]	6
	(surface tension, dynes/cm)					
Trial						
1	47.1	46.8	45.2	44.9	43.6	44.9
2	45.6	.....	44.6	45.2	43.6	44.6

[a] 1. Immediately after milking. 2. Degassed. 3. Storage 3–4 hr. at room temperature. 4. Heating to 60° C. and cooling to 20° C. 5. No. 4 after storage 3–4 hr. at 5° C. 6. No. 5 after heating and cooling as in No. 4.

[b] Only after this treatment was the fat in the solid state when the surface tension was measured.

that had been sensitized by homogenization at 105° F. (40.6° C.). Both tests were made after two days of storage of the heated milk at 400° F. (4.5° C.). The drop in surface tension from the most severe heating measured to the heating which just permitted organoleptic rancidity decreased from 3.0 dynes/cm at 184° F. (84.5° C.) × 0.03 sec. to 0.2 dynes/cm at 145° F. (62.8° C) × 400 sec. Surface tension values after not heating and after holding at 145° F. (62.8° C.) for 200, 300, 400, and 500 sec. were, respectively, 37.9, 43.7, 43.9, 44.4, and 44.5 dynes/cm (32). Average surface tension measurements (DuNuoy) at 60, 81, and 102° F. (15.6, 27.2, and 38.9° C.) were fitted by least squares to parabolas. Values for whole, homogenized, and skimmilk at 60 and 102° F. (15.6 and 38.9° C.), respectively, were 44.5, 47.2, 50.0, and 38.7, 42.0, 46.9 dynes/cm. The increasing values of calculated surface tension for skimmilk at 102° F. (38.9° C.) do not correspond to measured increases in any milk product by any

observer. Two parabolas or even two straight lines, one limited to the range of solid fat and the other to the range of liquid fat, might avoid this difficulty (71).

B. *Effects of homogenization.*

The respective surface tensions (dynes/cm) of skimmilk, either raw and unhomogenized, or pasteurized and homogenized three times at 200 atmospheres with 0, 4, 10, 20, and 30% melted butter oil, were 47.5, 44.2, 42.5, 43.5, 44.8, and 22.6 by a stirrup method, and 51.5, 50.6, 47.1, 47.8, 46.9, and 45.8 by a drop-number method (46). The expanded fat surface in homogenized milk withdrew surface-active materials (protein) from the plasma and raised the surface tension. If lipase was not inactivated by heat, it was activated by homogenization to produce fatty acids which lowered the surface tension. In milk containing less than 5 to 7% fat the latter effect predominated; whereas, with a higher per cent fat, aging was required to overcome the initially predominating former effect. The latter (enzyme) effect was destroyed by preheating the milk to 150° F. (64.6° C.) but not to 140° F. (60.0° C.) (23). Average respective values of surface tension (12 samples by DuNuoy method at 20° C.) after homogenization at

0, 500, 1500, and 2500	p.s.i. were
43.62, 39.61, 38.47, and 39.16	dynes/cm at 90° F. (32.2° C.) and
44.53, 44.97, 45.27, and 45.55	dynes/cm at 145° F. (62.8° C.)

after holding 30 min. (29, 68).

The surface tension of homogenized milk was increased by adding a concentrate of solids-not-fat or by using a pressure of over 3,000 p.s.i. Repeated homogenization or moderate previous addition of cream or water had very little effect, while preheating at 165° F. (73.9° C.) for 30 min. decreased the surface tension (20).

Effects of homogenization have been interpreted as follows: When milk is homogenized, the fat globule membrane becomes dispersed in the plasma and, perhaps, denatured. A new membrane is formed composed of the same or different enzymes, proteins, lipids, etc. proportional to their abundance, mobility, and ability to reduce surface tension. An increase of bound casein in the new membrane may cause the low curd tension in homogenized milk. The new membrane may have lecithin bound outside rather than inside the casein layer (23).

C. *Relations to developed flavors.*

Four aliquots of fresh whole milk, one unmodified, one with $CuSO_4$ added, one with vitamin C added, and one hand homogenized after addition of vitamin C, were diluted with water 10,000 and 100,000 times. Surface tensions of these dilutions (25° C. Wilhelmy) were measured at several surface ages. The induction time, before first decrease in surface tension, varied from a few seconds to 2 hr. These variations were not simply related to either the kind of treatment or the age of the treated sample (76).

Surface tensions (DuNuoy, 20° C. after 4 or 28 hr. at 4° C.) on 852 samples of milk distributed through about 1 yr. are shown in Table 7. Rancidity was

TABLE 7

Rancid flavor vs. surface tension of milk

Flavor class	No. samples	Dynes/cm	Std. dev.	Range
Not rancid	421	46.61	0.95	44.4–50.4
Slightly rancid	193	44.23	1.02	41.8–47.1
Rancid	238	40.74	2.52	32.3–46.1

estimated more conveniently and more exactly by changes in surface tension than by fat acidity determinations (25). Surface tension at age 24 hr. of milk samples from individual cows was used in a study of causes for natural or induced rancidity. Individuality of the cow, stage of lactation, and season of the year each appeared important in some instances, but could not be consistently used to predict the tendency of milk to develop rancidity (26). The rate of fall in surface tension of tenfold dilutions of fresh milk (vibrating jet 20° C.) was greater in those samples which developed oxidized flavor after storage for 3–5 days (74).

D. *Range.*

In addition to the ranges mentioned under previous headings, three other studies of the range of surface tension of milk should be mentioned. It was stated that the surface tension of pure milk has a fixed value of 52 dynes/cm and that dilution or separation had very little effect (39). A range of 48.8 to 55.3 averaging 50.4 dynes/cm (DuNuoy 18–20° C.), with values at 0° C. about 3 dynes/cm lower, was reported (8). Surface tensions of 1,351, 24-hr. samples from collecting stations averaged 55.9 dynes/cm. An unsuccessful effort was made to relate differences in surface tension with suitability of the milk for cheese-making (22).

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ANALYSIS OF MILK PHOSPHOLIPIDS BY CHROMATOGRAPHY AND INFRARED SPECTROPHOTOMETRY

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SUMMARY

Milk phospholipids were fractionated into the major phospholipid classes by successive elution from a silicic acid column with chloroform, acetone, methanol (20% and 40%) in chloroform, and methanol. Each fraction was analyzed by infrared spectrophotometry as well as by chemical methods. Potassium bromide disk spectra of milk cerebrosides, cephalins, lecithins, and sphingomyelins are presented and compared with pure reference compounds. Concentrations of each class were calculated from specific bands measured either in chloroform or in carbon disulfide solutions. Respective weight percentages of cerebrosides, cephalins, lecithins, and sphingomyelins were: 6, 35, 32, and 24. Evidence is given for the presence of minor amounts of other constituents, including carbohydrate-containing phospholipids, lysolecithins, and unidentified lipids. Advantages and limitations of the method at its present state of development are discussed.

Classical methods for separating and identifying individual phospholipids (3, 10, 23) are often laborious and seldom quantitative, and usually require large amounts of starting materials. An important advance was made in 1952, when Borgström (2) reported that phospholipids could be quantitatively separated from other lipids by adsorption chromatography with silicic acid columns. In his procedure, chloroform was used to elute the nonphospholipids, and methanol to elute all the phospholipids together in a single fraction. Subsequent investigators (5, 6, 13, 15, 17, 22) have shown that further fractionation of the phospholipids is possible by elution with chloroform-methanol mixtures. In general, many small fractions have been obtained and characterized chemically from their hydrolysis products.

Infrared spectrophotometry has been introduced to analyze the intact phospholipid molecules rather than their hydrolysis products. In 1957, Schwarz *et al.* (19) used a combination of infrared and chemical analyses to study numerous small fractions obtained from chromatography of brain lipids and rabbit-skin phospholipids. Nelson and Freeman (14) recently described a simplified method, using infrared spectrometry to determine cephalins, lecithins, and sphingomyelins in relatively small samples of human serum.

With the development of improved methods of lipid separation and analysis, there has been renewed interest in the composition of milk phospholipids; however, all their components have not yet been identified. A number of authors have used differential hydrolysis, either alone (12) or combined with chromatography (1, 4, 7, 18), to estimate the amounts of lecithins, cephalins, and sphingomyelins in various dairy products.

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Rhodes and Lea (18) obtained phospholipids from buttermilk and chromatographed them on a silicic acid column. Cephalin and lecithin fractions were recovered by elution with methanol-chloroform (3:7), and the remainder of the adsorbed choline-containing phospholipids by elution with methanol-chloroform-water (70:25:5). The cephalin and combined choline-containing fractions were examined by chromatographic and chemical methods. In addition to the major constituent phospholipids, minor amounts of lipid-bound inositol and plasmalogens were detected, but cerebrosides were not found. Deutsch *et al.* (4) chromatographed butter phospholipids on a silicic acid column and eluted four fractions with methanol-chloroform (1:4) and methanol. Lecithins and cephalins were identified by paper chromatography, and sphingomyelins by selective saponification. The researchers suggested that cerebrosides were present among the unidentified constituents.

This paper describes a comparatively simple procedure, in which the mixed phospholipids of milk were fractionated into the major phospholipid classes by column chromatography and each fraction analyzed by infrared spectrophotometry as well as by chemical methods. The spectra of milk cerebrosides, cephalins, lecithins, and sphingomyelins are presented and compared with the spectra of pure reference compounds.

EXPERIMENTAL PROCEDURE

Preparation of phospholipid samples. The following reference compounds were used: phrenosin—H. E. Carter; sphingomyelin (spleen)—S. J. Thannhauser; L-α-(dioleoyl)-cephalin (synthetic)—E. Baer; and L-α-(dimyristoyl)-lecithin (synthetic)—La Motte Chemical Products Co.

The method used to isolate mixtures of phospholipids from spray-dried buttermilk has been described in detail (20). Briefly, the lipids were extracted with ethanol, ether, and chloroform, and most of the phospholipids were separated from the other lipids by precipitating them twice with acetone. Acetone-soluble phospholipids were recovered by chromatography with silicic acid. The two phospholipid fractions were combined and washed twice to remove nonlipid contaminants.

Chromatographic separation. For a 1-g. sample, 75 g. of a 2:1 mixture of silicic acid and celite was used in a column (Fischer and Porter Co., Hatboro, Pa.) 2.5 cm. in diameter and 60 cm. long that was fitted with a fritted glass disk and an "Ultramax" Teflon valve. The silicic acid (Mallinckrodt, precipitated) was washed twice with water to remove the smaller particles that remained in suspension. The celite (Johns-Manville, 100-200 mesh) was washed successively with 10 *N* hydrochloric acid, water, and methanol. The mixture of silicic acid and celite was heated at 125° C. for 16 hr. before use and slurred into the column with methanol. A 5-mm. layer of anhydrous sodium sulfate (between filter paper disks) and a perforated porcelain disk were placed on top of the silicic acid–celite column to remove any trace of moisture from the eluants and to protect the top of the adsorbent. Before use, the column was washed successively

with 500-ml. volumes of methanol, acetone, and chloroform. All solvents were reagent grade.

The phospholipid sample was added to the column in 200 ml. of chloroform. Elution was carried out as shown in Table 1. Successive eluants were applied just before the last of the preceding eluant entered the column, and the receiver was changed at the same time. The flow rate varied somewhat at different stages, but averaged about 4 ml. per minute.

Each eluate was reduced in volume in a rotating evaporator under partial vacuum at 50° C. before being transferred into a tared volumetric flask fitted with a S/T stopper. After the remainder of the solvent was evaporated with a stream of nitrogen, the flask was reweighed. Then each flask was filled to its calibration mark with chloroform and flushed with nitrogen. To prevent evaporation, the stopper and top of each flask were covered with Saran film. All samples were stored at 0–5° C. and analyzed within a few days of preparation.

Chemical methods of analysis. Lipid phosphorus was determined as previously described (21), and total nitrogen by the micro method of Ma and Zuazaga (11). The anthrone (16) and ninhydrin reactions (9), respectively, were used as semiquantitative tests for reducing sugars and amino nitrogen. All chemical data reported are averages of at least two determinations.

Infrared measurements. Potassium bromide disks were prepared as follows: An aliquot containing 0.8–2.1 mg. of phospholipid was evaporated in a mortar under a heat lamp with a stream of nitrogen, 400 mg. of potassium bromide (Harshaw, infrared quality) was ground thoroughly with the sample, and the mixture was transferred to an evacuable ½-in. steel die. The die was sealed by applying slight pressure on the plunger with a Carver press, and was then evacuated with a vacuum pump. The pressure exerted on the die was gradually increased to 17,000 lb., which was held for 15 min. The disks were placed in a vacuum desiccator and their spectra were subsequently recorded in the range of 2.5 μ to 15 μ with a Perkin-Elmer Model 137 Infracord spectrophotometer.

Spectra recorded from potassium bromide disks have advantages for qualitative investigations of phospholipids, because they are unobscured by solvent absorption, but the disks are somewhat cloudy for some samples, making quantitative estimations difficult. For this reason, the authors prefer to base quantitative estimations on bands measured in solution.

Infrared absorption spectra of the samples in solution were recorded from 2 μ to 11 μ with a Baird Associates Model B spectrophotometer equipped with a sodium chloride prism. These spectra were obtained from either chloroform or carbon disulfide solutions (depending on solubility) containing 6.8–7.1 mg/ml of sample. The absorption cell used had an optical path length of 0.9 mm.

Absorbance A at each specified absorption maximum was calculated as $A = \log_{10} \frac{T_o}{T_S}$, where T_o is transmittance of the solvent and T_s is transmittance of the sample solution. The amounts of cephalins, lecithins, and sphingomyelins were calculated from available calibration data (14).

TABLE 1

Chemical analyses of fractions from chromatography of milk phospholipids

Fraction	Eluant	Eluant volume	Fraction weight	Lipid phosphorus		Total nitrogen		Amino nitrogen	Reducing sugar	Principal components
		(ml.)	*(mg.)*	*(%)*	*(mg.)*	*(%)*	*(mg.)*			
I	Chloroform	400	84	0.04	0.0	0.20	0.2		−	Nonphospholipids
II	Acetone	500	71	0.34	0.2	1.90	1.4		+++	Cerebrosides
IIIa	20% Methanol in chloroform	100	62	3.05	1.9	1.91	1.2	++	+	Cephalins
IIIb	20% Methanol in chloroform	200	206	3.92	8.1	1.95	4.0	+++	−	Cephalins
IIIc	20% Methanol in chloroform	200	30	4.06	1.2	2.25	0.7	+++	−	Cephalins
IVa	40% Methanol in chloroform	100	42	3.84	1.6	2.48	1.0	++	+	Cephalins
IVb	40% Methanol in chloroform	300	145	4.10	6.0	2.01	2.9	+	−	Lecithins
IVc	40% Methanol in chloroform	600	284	3.86	11.0	2.41	6.8	−	-	Lecithins and sphingomyelins
Va	Methanol	100	85	4.00	3.4	3.47	3.0	−	−	Sphingomyelins
Vb	Methanol	400	51	3.92	2.0	3.28	1.7	−	−	Sphingomyelins
Total recovered			1,060		35.4		22.8			
Total applied			1,003	3.72	37.2	2.12	21.2			

RESULTS AND DISCUSSION

Infrared spectra of reference phospholipids. Potassium bromide disk spectra of phrenosin, L-α-(dioleoyl)-cephalin, L-α-(dimyristoyl)-lecithin, and sphingomyelin are shown (Figure 1). A portion of the spectrum of cephalin in carbon disulfide solution is included in Figure 1-B. Both the phrenosin and sphingo-

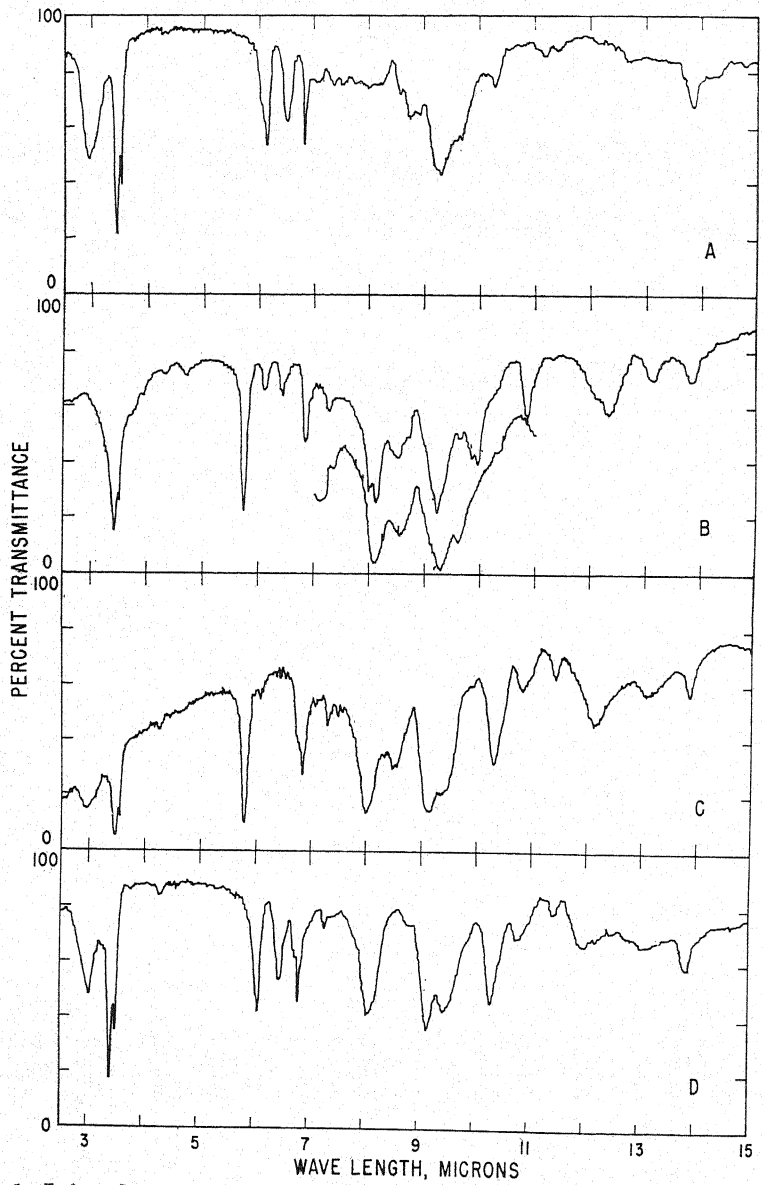


FIG. 1. Infrared spectra of phospholipid reference samples in 400-mg. potassium bromide disks. *A*. Phrenosin, 0.8 mg. *B*. L-α-(dioleoyl)-cephalin: Upper curve—0.9 mg. in KBr, lower curve—10 mg/ml in carbon disulfide. *C*. L-α-(dimyristoyl)-lecithin, 1.5 mg. *D*. Sphingomyelin, 0.8 mg.

myelin spectra correspond to those found by Schwarz *et al.* (19). Sphingolipids, such as sphingomyelins, phrenosins, and other cerebrosides, can be distinguished from other lipids by the strong absorption bands at 6.1 μ and 6.5 μ that are characteristic of monosubstituted amides (-CONH-). (Note that weak bands of uncertain origin appear at these positions in the spectrum of cephalin also.) The sphingomyelin spectrum differs from the phrenosin spectrum, in that the former has a strong band at 8.1 μ, a doublet at about 9.1–9.4 μ, and a stronger band at 10.3 μ.

The spectra of both cephalin and lecithin have, at 5.8 μ, a strong ester carbonyl band that is absent in sphingolipid spectra. Lecithins can be distinguished from cephalins by a strong band at 10.3 μ that is absent in cephalins. The disk spectrum of L-α-(dioleoyl)-cephalin in Figure 1 shows weak bands at about 6.1 μ and 6.4 μ, some absorption at about 9.6–10 μ, and a band at 10.8 μ. Schwarz *et al.* also observed in cephalins, weak absorption at 6.1 μ and a stronger band at 9.8 μ. However, the bands in the 9.8 μ and 10.8 μ regions must be associated with the physical state of the sample, because they do not appear in the solution spectrum of the reference cephalin (Figure 1-B). The spectrum of the reference lecithin corresponds to that reported by Schwarz *et al.* except that, in our curve, the band at about 7.3 μ is much weaker and there is less indication at 6.1 μ for the presence of small amounts of water in the sample.

Analysis of milk phospholipid fractions. During this investigation, five different samples of milk phospholipids (20) were analyzed by silicic acid chromatography and infrared spectrophotometry, but the results for only one sample are presented. These are representative of the earlier work and are more complete.

Weights and chemical analyses of the ten fractions obtained by chromatographic separation of mixed phospholipids from buttermilk are listed (Table 1). The total weight recovered indicates that the eluting solvent was not completely removed from some of the fractions before they were weighed. However, considering the numerous possibilities of error, the recoveries of both lipid phosphorus and total nitrogen were satisfactory. The chemical data indicate that the order of elution was cerebrosides, cephalins, lecithins, and sphingomyelins.

Each fraction was further characterized by infrared spectra and absorbance measurements at specific wave lengths. Potassium bromide disk spectra of Fractions II to Vb are presented (Figures 2, 3, 4, and 6). The composition of each fraction is discussed in the order of elution.

Fraction I. Essentially no phospholipids were eluted by chloroform. The infrared spectrum of this sample did not reveal any significant amounts of cholesterol, glycerides, or fatty acids, but indicated the presence of phthalate and, possibly, carotenoids. When an aliquot of this fraction was dissolved in iso-octane and examined in the ultraviolet region, an absorption peak was found at 275 mμ. Di-*n*-butyl phthalate absorbed strongly at the same wave length. Fraction I probably consisted mostly of phthalate plasticizer that had been present as a contaminant in the original crude phospholipid sample. This fraction was not included in subsequent calculations of the amounts of each phospholipid class.

Fraction II—(Figure 2). The spectrum for this sample, which was eluted

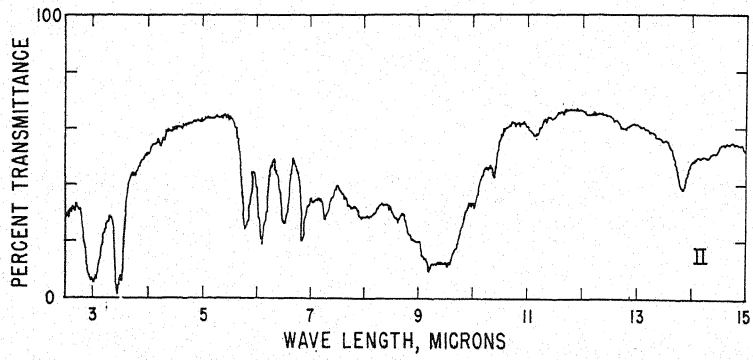


FIG. 2. Infrared spectrum of Fraction II (mostly cerebrosides) from chromatography of milk phospholipids, 2.1 mg. in 400 mg. potassium bromide.

with acetone, resembles in most respects the spectrum of phrenosin (Figure 1-A) and exhibits the characteristic amide absorption bands at 6.1 μ and 6.5 μ. Absorption in the 9–9.5 μ region is attributed to C=O bonds in the sugar part of the cerebrosides.

Unfortunately, calibration data are not available for the 6.1 μ band of a pure cerebroside. However, when the absorption coefficient of sphingomyelin was used, the calculated cerebroside concentration was about 80%. The amount found by chemical analysis was 96%, but less confidence can be placed in this result, because anthrone is not specific for cerebrosides.

The carbonyl ester band at 5.8 μ indicates the presence of cephalins in this fraction. But if cephalins have an average phosphorus content of about 4.0%, this fraction could contain only about 7% cephalins. The concentration of cephalins was 35–40% when calculated from the 5.8 μ band of Fraction II dissolved in chloroform, but excessive absorption at 5.8 μ could be attributed to the presence of small amounts of nonphospholipid esters not completely eluted by chloroform.

In view of the above considerations, and the probable errors involved (14), the following composition for Fraction II was tentatively accepted: cerebrosides 80%, cephalins 10%, unidentified lipids 10%.

Rhodes and Lea (18) found no cerebrosides in the choline-containing phospholipids of milk, but their report did not indicate that they tested their cephalin fraction for reducing sugars. Since, in our work as well as in that of Schwarz *et al.* and Deutsch *et al.*, cerebrosides were eluted before cephalins, it seems possible that the cephalin fraction of Rhodes and Lea may have contained some cerebrosides.

Fractions IIIa, IIIb, and IIIc—(Figure 3). Most of the cephalins present in the original mixture of milk phospholipids were eluted in these fractions with 20% methanol in chloroform. The term cephalins includes both phosphatidyl ethanolamines and phosphatidyl serines, since they are presumably both eluted by this solvent and are virtually indistinguishable from one another by their infrared spectra.

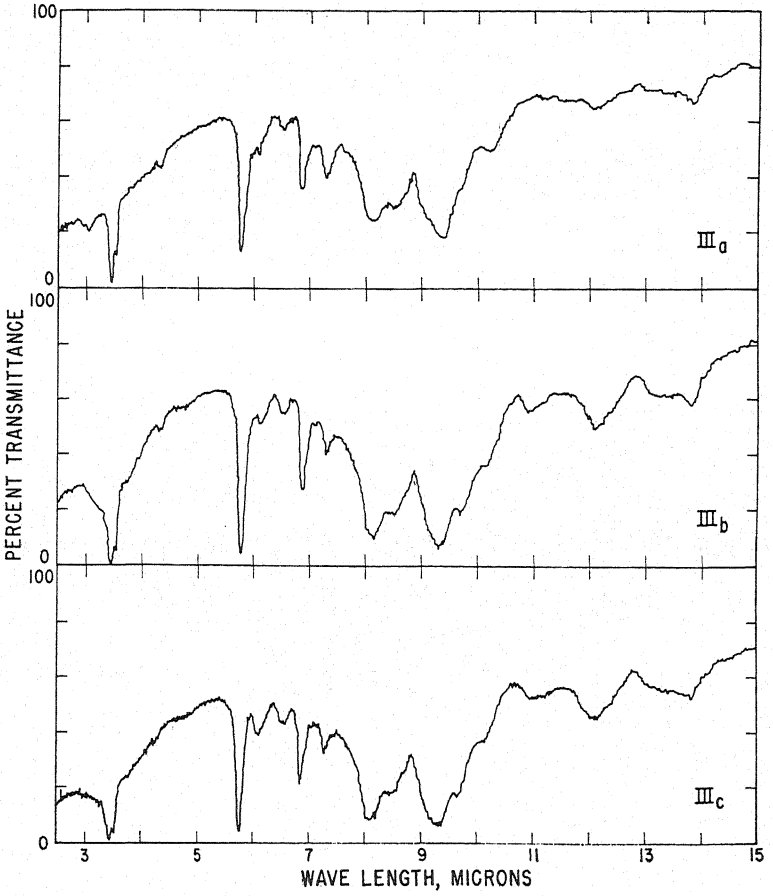


FIG. 3. Infrared spectra of Fractions IIIa, IIIb, and IIIc from chromatography of milk phospholipids in 400-mg. potassium bromide disks. *IIIa.* Cephalins and cerebrosides mixture, 2.1 mg. *IIIb.* Cephalins, 1.2 mg. *IIIc.* cephalins, 1.8 mg.

The disk spectra for the three samples are similar and correspond in most regions to those of the reference cephalin. They are also similar to the spectra for L-α-(dimyristoyl)-cephalin and rabbit skin cephalin (19). The absence of a 10.3 μ band shows that no lecithins or sphingolipids were present in Fractions IIIb and IIIc.

The concentration of cephalins in Fraction IIIa was 98% when calculated from the 5.8 μ band of its chloroform solution, but only around 80% on the basis of phosphorus content. Therefore, an average value of 90% cephalins was accepted. Since the spectrum shows minor absorption at 10.3 μ and the sample gave a positive anthrone reaction, the remaining 10% was probably cerebrosides.

Fraction IIIb was essentially all cephalins on the basis of its absorptivity at 5.8 μ in carbon disulfide. This is confirmed by the percentages of lipid phosphorus and total nitrogen, the strongly positive test for amino nitrogen, and the negative test for cerebrosides.

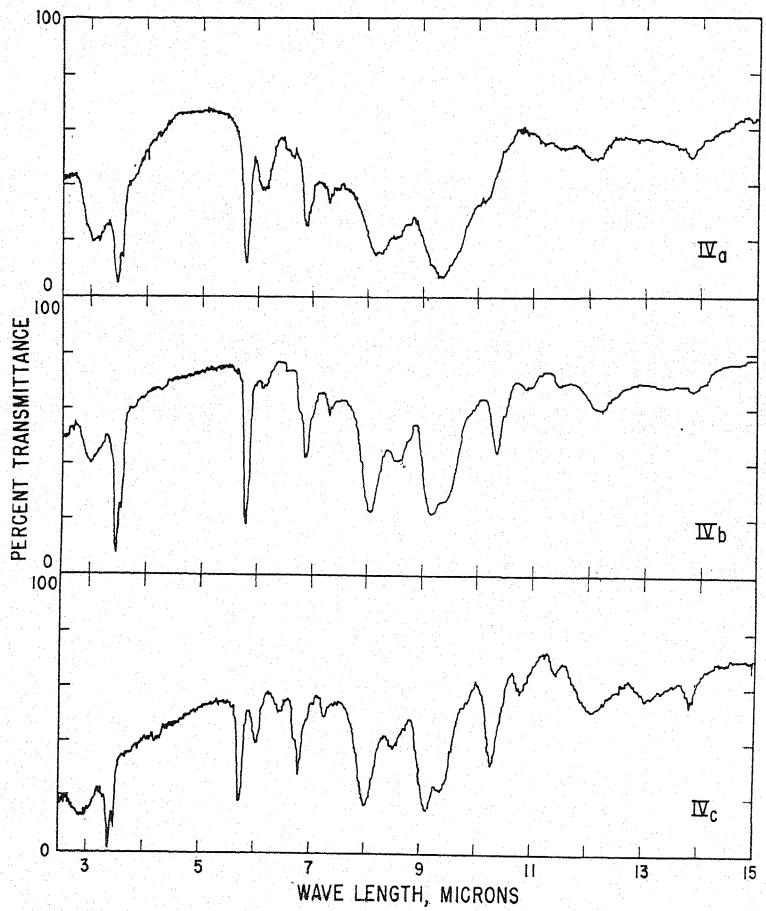


FIG. 4. Infrared spectra of Fractions IVa, IVb, and IVc from chromatography of milk phospholipids, in 400-mg. potassium bromide disks. *IVa.* Mostly cephalins, 2.1 mg. *IVb.* Lecithins, 1.0 mg. *IVc.* Lecithin and sphingomyelin mixture, 1.1 mg.

Fraction IIIc was also calculated to be all cephalins, although the total nitrogen percentage is somewhat higher than the theoretical percentage for cephalins.

Fractions IVa, IVb, and IVc—(Figure 4). The remainder of the cephalins, all the lecithins, and part of the sphingomyelins were eluted in these fractions with 40% methanol in chloroform.

The spectrum of Fraction IVa resembles those of the reference cephalin (Figure 1-B) and of L-α-(dimyristoyl)-cephalin (19), except that its ratio of absorption at 9.3 μ to 8.1 μ is greater. Absence of a band at 10.3 μ shows that there were no lecithins or sphingomyelins present. On the basis of absorption measured at 5.8 μ in carbon disulfide, the concentration of cephalins was calculated as 90%. However, Fraction IVa contained 2.48% nitrogen and gave a positive sugar reaction. Therefore, both the infrared and chemical data indicate that the remaining unidentified 10% of this fraction included sugar-containing lipids—possibly some type of cerebroside or phosphoinositide. Hanahan *et al.*

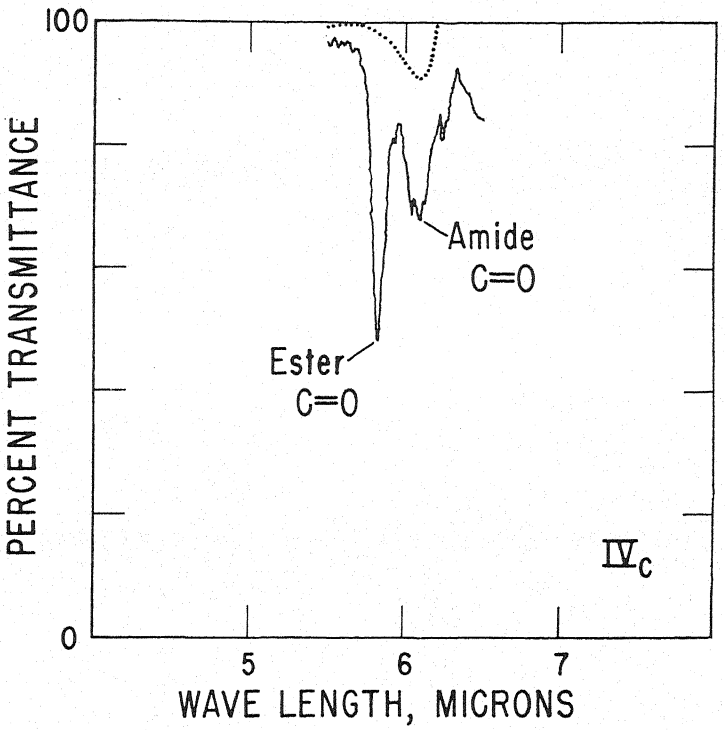


FIG. 5. Example of infrared absorption bands used in calculating lecithin and sphingomyelin in mixed fractions. (Curve shown is for Fraction IVc, 5.68 mg/ml in chloroform.) Dotted line is solvent transmittance.

(6) reported that inositol-containing phospholipids were eluted from silicic acid with 40% methanol in chloroform, and Rhodes and Lea (18) found inositol in a milk phospholipid fraction eluted with 30% methanol in chloroform.

The spectrum for Fraction IVb corresponds very closely to that of the reference lecithin (Figure 1-C). The total amount of lecithins present was 100% and 94%, respectively, when calculated from the absorptivities at 9.15 μ and 10.3 μ. Since this fraction also gave a weakly positive amino nitrogen test and a negative sugar test, a composition of 95% lecithins and 5% cephalins was accepted.

Fraction IVc was a mixture of lecithins and sphingomyelins. This is shown by the strong band at 10.3 μ common to both phospholipids, the amide group bands at 6.1 μ and 6.5 μ found in sphingolipids, and the ester carbonyl band at 5.8 μ attributed to the lecithins present. The concentrations of each component were calculated from the absorbances at 5.8 μ and 6.1 μ, measured in chloroform (Figure 5), using the following simultaneous equations:

$$C_{\text{Lecithins}} = k_1 A_{5.8} + k_2 A_{6.1},$$
$$C_{\text{Sphingomyelins}} = k_3 A_{5.8} + k_4 A_{6.1}.$$

Values for the constants k_1, k_2, k_3, and k_4 were calculated from calibration data (14). Fraction IVc contained 60% lecithins and 40% sphingomyelins. On the

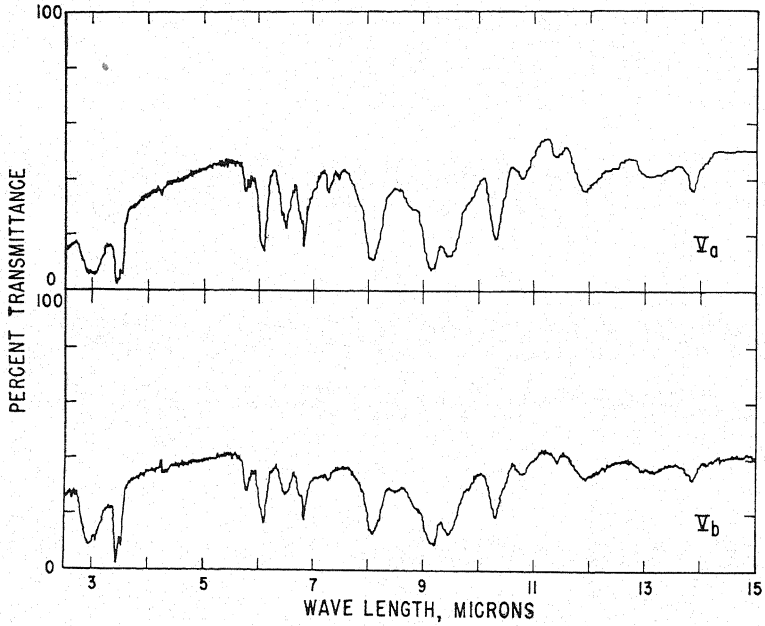


FIG. 6. Infrared spectra of Fractions Va and Vb from chromatography of milk phospholipids, in 400-mg. potassium bromide disks. *Va.* Sphingomyelins, 1.7 mg. *Vb.* Mostly sphingomyelins, 1.0 mg.

basis of absorption measured at 10.3 μ, these two classes of phospholipids accounted for all of the fraction.

Fractions Va and Vb—(Figure 6). These last two fractions were eluted with methanol, and consisted mostly of sphingomyelins. Their spectra correspond with that of the reference sphingomyelin (Figure 1-D), except for the presence of minor carbonyl ester bands near 5.8 μ. The composition of each fraction was calculated, as above, from the absorbances at 5.8 μ, 6.1 μ, and 10.3 μ measured in chloroform.

Fraction Va contained 95% sphingomyelins and 5% lecithins, and the weight calculated at 10.3 μ accounted for all of the sample.

Fraction Vb contained 85% sphingomyelins and 15% lecithins when calculated from the equations. However, the total percentage of phospholipid was 110% from the absorptivity at 10.3 μ. The presence of lysolecithins, which should be eluted after sphingomyelins (15), could account for both the presence of the ester band at 5.8 μ and the increased absorptivity at 10.3 μ. There is no spectroscopic characteristic by which lysolecithin could be distinguished from lecithin in such a mixture. Since it is considered probable that lysolecithins could be present, a composition of 85% sphingomyelins, 10% lysolecithins, and 5% lecithins was tentatively accepted for this fraction.

Composition of milk phospholipids. The proportions of the major phospholipid classes in mixed milk phospholipids were calculated from the estimated compositions of the above fractions, and are compared with data recently reported

TABLE 2

Comparison of major phospholipid classes found in milk by various investigators

Phospholipid class	Present study		Baliga and Basu (1)[a]	McDowell (12)[a]	Rhodes and Lea (18)	Koops (7)	Deutsch *et al.* (4)
	(wt. %)	*(moles %)*[a]	*(moles %)*				
Lecithins	32	31	29	28	33	30	33
Cephalins	35	37	47[b]	43	39	40	38
Sphingomyelins	24	23	24	29	19	25	23
Cerebrosides	6	6	....	....	Not found	Not found	Found

[a] Assuming following average molecular weights: lecithins, 806; cephalins, 744; sphingomyelins, 831; cerebrosides, 826.
[b] By difference.

by others (Table 2). The percentages of lecithins, cephalins, and sphingomyelins found in the present study agree quite closely with those of Rhodes and Lea (18), Koops (7), and Deutsch *et al.* (4). The appreciable amount of cerebrosides found is consistent with the earlier work of Kurtz and Holm (8).

It is concluded that a combination of chromatography and infrared spectrophotometry is very useful for studying milk phospholipids. Chromatographic fractionations can be followed and the principal components readily determined from the spectra. Accuracy is influenced by variations in the extinction coefficients of various lecithins etc., and by the composition of the fractions examined, but is probably equal to that attained by microchemical analysis (14, 19). Only comparatively small samples are necessary for infrared spectrophotometry, and they may be recovered if desired. Isolation and quantitative analysis of constituents occurring in minor amounts, such as lysophospholipids, inositol-containing phospholipids, and plasmalogens, would require both a more elaborate fractionation procedure than that used in the present study and highly purified reference compounds that are not yet readily available.

ACKNOWLEDGMENTS

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STUDIES ON CASEIN. II. THE CARBOHYDRATE MOIETY OF CASEIN

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SUMMARY

The carbohydrate in casein has been estimated by hydrolysis of the protein with a mixture of dilute hydrochloric acid and a cation exchange resin, passage of the hydrolysate through a cation exchange column to remove interfering substances, and the measurement of the colour produced by the reaction of the liberated sugar with an anthrone reagent. The sugar contents of whole casein (Warner), alcohol-extracted casein, α-casein, β-casein, and γ-casein determined in this manner were approximately 3.8, 1.8, 1.5, 0.8, and 0.9 mg/g, respectively.

The individual sugars in the casein preparations were determined quantitatively by filter paper chromatography. Whole casein contained galactose (1.84 mg/g), glucose (1.11 mg/g), and mannose (0.53 mg/g). Alcohol-extracted whole casein contained galactose (1.34 mg/g) and mannose (0.56 mg/g) and α-casein also contained galactose (0.75 mg/g) and mannose (0.48 mg/g). β-casein contained only galactose (0.52 mg/g) and γ-casein contained only galactose (0.48 mg/g).

Sorensen and Haugaard (20) applied the orcinol reaction to the determination of carbohydrate groups in proteins. From the time-curve of the orcinol reaction, they concluded that Hammersten casein contained 0.31% galactose. Sorensen (19) suggested later that the carbohydrate component of casein was a mixture rather than a single substance. This was confirmed by Mesamune and Maki (14), who detected mannose, galactose, and glucosamine in acid hydrolysis of casein by the use of partition chromatography on paper.

Koiw and Gronwall (10) developed a method for staining protein-bound carbohydrate after electrophoresis of serum in filter paper. Their method involved the periodic acid–Schiff technique developed by McManus (16) and by Hotchkiss (7).

Glegg *et al.* (4) pointed out that the hydroxyamino acids, serine and threonine, at the end of the protein chain, and hydroxylysine at any position in the chain, would give rise to a periodic acid–Schiff reaction. However, the terminal amino acids of α- and β-casein have been shown to be restricted to lysine and arginine (17), and it is highly unlikely that casein contains hydroxylysine. It is probable, therefore, that the staining of casein by the periodic acid–Schiff technique is due to the presence of carbohydrate.

MacRae and Baker (12, 13) separated the α-, β-, and γ-components of the casein complex by filter paper electrophoresis and determined the positions of the protein bands by staining with Azocarmine B. Duplicate strips of the paper were stained for carbohydrate according to the method of Koiw and Gronwall (10). They observed staining only at the position of the α-component. In another experiment, in which equal amounts of purified α-, β-, and γ-casein were applied to the paper, the proteins subjected to electrophoresis, and then stained for carbohydrate, staining was observed only at the position of the α-casein band.

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This paper deals with (a) the development of a method for the estimation of protein-bound carbohydate and the application of the method to the determination of the carbohydrate content of casein and (b) the quantitative estimation of the individual sugars in casein by paper chromatography.

MATERIALS

Whole casein was prepared from a composite sample of milk (Macdonald College herd) according to the method of Warner (21). The casein was thoroughly washed with water and dried with alcohol and ether (Preparation No. 1).

Alcohol-extracted casein was prepared by extraction of whole casein (Preparation No. 1) with 95% ethanol in a Soxhlet extractor for 96 hr.

α- and β-casein were prepared from whole casein (Preparation No. 1) by the method of Hipp *et al.* (6) and γ-casein was prepared by the method of Hipp *et al.* (5).

EXPERIMENTAL METHODS

Determination of sugar by the anthrone reaction. Preliminary experiments. It was essential at the outset to find a method for the hydrolysis of the protein which would liberate the sugars from the protein, and at the same time would not lead to sugar destruction.

Casein was hydrolyzed with hydrochloric acid (4 *N*) for periods of time ranging from 1 to 8 hr. The separate hydrolysates were treated with the anthrone reagent (3) before and after the amino acids and amino sugars had been removed from the hydrolysates by means of Dowex 50 (1). The absorbances of the various solutions were measured at wave lengths ranging between 540 and 680 mμ by means of a Beckman Spectrophotometer (Beckman Model DU). Figure 1 shows typical curves obtained with the various solutions [A—hydrochloric acid (4 *N*) hydrolysate before treatment with resin; B—water eluate of Dowex 50 column through which the hydrochloric acid (4 *N*) hydrolysate passed; C—acid eluate of Dowex 50 column through which the hydrochloric acid (4 *N*) hydrolysate had passed]. It will be observed that the characteristic peak at 620 mμ which is given by glucose was not given by the hydrolysate, either before or after treatment with cation exchange resin.

Adopted procedure. Casein (100 mg.) and 5 ml. of a cation exchange resin suspension (one part Dowex 50, 200-400 mesh, in two parts of 0.05 *N* hydrochloric acid) were placed in a Pyrex test tube (13 by 100 mm.). The tube was sealed and then heated in a rotary oven at 100° C. for 16 hr. At the end of the heating period the tube was cooled and opened. The contents of the tube were placed on the top of a Dowex 50 column (10 by 30 mm.) which was in the hydrogen form. The eluate was collected and the column was washed with 10 ml. of distilled water. The eluates were combined and labelled "water eluate." The column was then washed with hydrochloric acid (10 ml., 2 *N*) followed by distilled water (2 ml.). These eluates were combined and labelled "acid eluate." Traces of amino compounds which might be contained in the water eluate were removed by passage of the solution again through the column. The column was

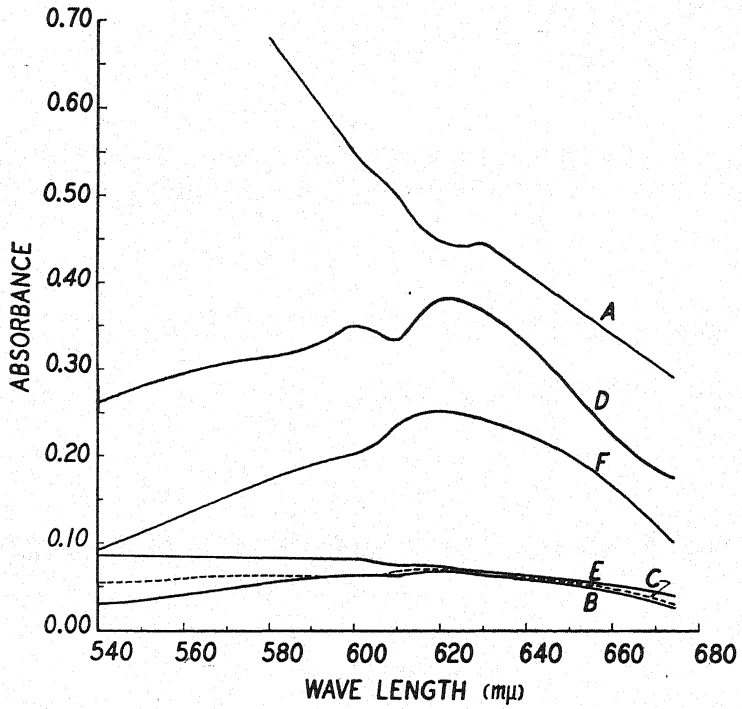


FIG. 1. The effects of methods of hydrolysis and resin treatment on the absorbance of casein hydrolysates after treatment with anthrone reagent.

A—Hydrochloric acid (4 *N*) hydrolysate before treatment with Dowex 50; B—hydrochloric acid (4 *N*) hydrolysate after treatment with Dowex 50; C—acid eluate of Dowex 50 column through which the hydrochloric acid (4 *N*) hydrolysate had passed; D—Solution S_1 (water eluate from resin hydrolysis); E—Solution S_2 (acid eluate from resin hydrolysis); F—glucose standard.

then washed with water (8 ml.) and the eluates were combined and the volume adjusted at 25 ml. This solution (S_1) was assumed to contain the sugar which was to be determined. The column was then washed with hydrochloric acid (8 ml., 2 *N*) and this eluate was combined with the "acid eluate" and the volume adjusted at 25 ml. This solution (S_2) was assumed to contain the amino sugars.

The sugar was determined by the method described by Fairbairn (3). Two, 2-ml. portions of the water eluate (S_1) were each treated with 10 ml. of the anthrone reagent. The colour was developed by heating the mixture in a boiling water bath for 12 min. The solutions were cooled and their absorbances were measured at 620 mμ by means of an Evelyn colorimeter. The interval between the end of the heating period and the time of measurement of absorbances was 20 min.

A standard curve was prepared by plotting the absorbances (620 mμ) of standard glucose solutions (5, 10, 20, 30, 40 μg/ml) which had been treated with the anthrone reagent, against the concentrations (μg/ml) of the standard solutions. The sugar concentration in the water eluates and, hence, the percentage sugar

(reported as glucose) in the casein sample, was obtained by referring the average of the absorbances of the duplicate samples of the unknown solution to the standard curve.

A sample of casein was hydrolyzed with the resin mixture, the hydrolysate passed through the resin column, and the water eluate (S_1) treated with the anthrone reagent. The resin column was then eluted with hydrochloric acid (2 *N*) and the eluate (S_2) treated with the anthrone reagent. The absorbances of the two solutions (S_1, S_2) were measured at various wave lengths (540-650 mμ) by means of a Beckman Spectrophotometer (Model DU). Figure 1 shows that the characteristic peak at 620 mμ obtained with the glucose solution and the water eluate was not obtained with the acid eluate (S_2).

Effects of period of hydrolysis. Samples of casein were treated with the Dowex 50 hydrochloric acid mixture as outlined previously, for periods ranging from 6 to 36 hr., and the sugars were determined on the resulting hydrolysates. The results for hydrolytic periods of 6, 10, 16, 24, and 36 hr. were 0.7, 0.67, 0.80, 0.60, and 0.63 mg. of sugar per gram of casein, respectively.

Determination of hexosamine. Ten milliliters of the combined acid eluates (S_2), obtained in the procedure for the determination of sugar by the anthrone reaction, were evaporated to dryness in a vacuum desiccator to remove hydrochloric acid. The residue was dissolved in 5 ml. of distilled water. Hexosamine was determined (1) in triplicate on 0.5-ml. portions of this solution.

Chromatographic methods. Identification of sugars. The method of McFarren *et al.* (15) was employed for the separation of galactose, glucose, and mannose by filter paper chromatography. The sugars were stained on the paper by use of aniline hydrogen phthalate (18).

Quantitative determination of the sugars. The same solvent system and staining agent were used as for the qualitative identification of the sugars. The procedure for the application of the standards and unknown solutions to the paper was essentially that described by Baker and Khan (2). However, instead of employing equal quantities of different dilutions of the standard as in the previous work, different quantities (4, 6, 11, 14, 18 μl.) of a standard solution containing 1.67 μg/ml of each sugar (galactose, glucose, mannose) were applied to each paper. The solution was applied in 1-2-μl. quantities and the spots were dried between application. Four spots containing the unknown were applied to each paper. Twenty microliters of the appropriate hydrolysates was used for each spot in the analysis of whole casein and 30 μl. was used for each spot in the analyses of the α-, β-, and γ-casein.

The procedure for measurement of the maximum colour density of the spots, preparation of standard curves, and evaluation of the results have been described previously (9). No filter was employed in the measurement of the maximum colour density.

RESULTS AND DISCUSSION

The sugar content of casein. Whole casein and also the α-, β-, and γ-fractions of the casein complex were analyzed for their sugar contents. The analyses were

performed on duplicate hydrolysates (Series 1, Series 2) and each result reported in Table 1 represents the average of duplicate determinations on each hydrolysate.

The hexosamine content of cow casein reported by Johansson and Svennerholm (8) was 1.8 mg/g and by Anastassiadis and Common (1), 1.66 mg/g. The total carbohydrate (hexosamine, hexose, sialic acid) of cow casein reported by Johansson and Svennerholm (8) was 8.0 mg/g.

TABLE 1

Sugar and hexosamine content of casein[a]

Sample	Sugar (by anthrone)		Hexosamine	
	Series 1	Series 2	Series 1	Series 2
	(mg/g)			
Whole casein (Warner)	3.81	3.88	2.24	2.16
Whole casein (alcohol-extracted)	1.77	1.76	1.64	1.68
α-casein	1.55	1.48	2.12	2.11
β-casein	0.75	0.79	1.15	1.10
γ-casein	0.87	0.86	2.37	2.48

[a] Based on 15% nitrogen.

Identification of the sugars in casein. Duplicate samples of whole casein (150 mg.) were heated with the resin suspensions (5 ml.), and the resulting hydrolysates were treated with the resin column by the adopted procedure described above. The duplicate water eluates (S_1) were combined and then were evaporated to dryness in vacuo. The residue was dissolved in 0.3 ml. of distilled water and 30 μl. of the resulting solution (S_3) was applied to the paper in one spot. The chromatogram was prepared as described in the section on Methods. The results showed that either the main sugar constituent was an unknown sugar with an Rf value less than that given by the galactose standard, or that it was galactose which was giving an Rf value less than that given by the standard. Two other smaller spots were observed on the paper, one with a slightly lower Rf value than the mannose standard and another with a slightly lower Rf value than the glucose standard.

In another experiment, a mixture of 10 μl. of the standard mixture (5 μg/ml of each sugar—galactose, glucose, mannose) and 30 μl. of solution S_3 was applied to the paper and the chromatogram was prepared. Three spots with Rf values approximately the same as those obtained with S_3 alone were observed. This experiment showed (a) that the presence of the hydrolysate reduced the Rf values of the sugars and (b) that the three spots which were obtained with S_3 corresponded to galactose, glucose, and mannose.

The interfering substances in S_3 were removed from the solution by passage of Solution S_3 through a column (13 by 76 mm.) of Permutit S in the carbonate form. The column was washed with water after the passage of S_3 through the column and the combined eluates and wash water evaporated to dryness in vacuo. The residue was dissolved in 300 μl. of distilled water. Chromatograms were prepared with this solution (S_4), and three spots having Rf values identical to those given by galactose, glucose, and mannose standards were obtained.

Solutions (S_4) were prepared using alcohol-washed whole casein, α-, β-, and γ-casein. The chromatograms obtained with these solutions and also with the solution (S_4) prepared from whole casein are shown in Figure 2.

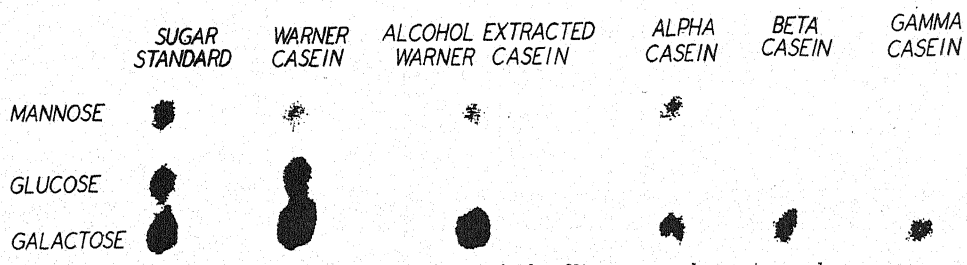


FIG. 2. Identification of sugars in casein by filter paper chromatography.

Quantitative estimation of the individual sugars in casein. The sugars in S_4 were determined quantitatively by filter paper chromatography. Table 2 shows the results obtained with whole casein, alcohol-extracted whole casein, α-, β-, and γ-casein. Each sugar was determined on duplicate hydrolysates and each result represents the average of eight determinations.

Sugar recovery. Duplicate samples (0.3 ml.) of a sugar solution containing 1.66 μg/ml of each sugar (galactose, glucose, mannose) were treated in exactly the same manner as was the casein in the preparation of S_4. The sugars in the resulting solution were determined by the filter paper chromatography method and the following recoveries were realized: galactose, 78.0%; glucose, 74.4%; mannose, 75.8%.

When electropherograms of whole casein were examined by the periodic acid–Schiff technique, a positive reaction was observed in the α-casein zone but not elsewhere. When electropherograms of α, β-, or γ-casein (prepared by precipitation methods) were examined in the same way, only α-casein gave a positive reaction (13). These observations were confirmed in the course of the present study. By themselves, these observations might lead to the supposition that the carbohydrate of casein is confined to the α-fraction. However, the work now reported shows clearly that sugars can be detected in hydrolysates of all three fractions when the hydrolysis is performed with a resin–dilute acid mixture.

Leblond *et al.* (11) have suggested that freely reactive 1,2-glycol groups in carbohydrate-protein complexes may be partially responsible for the positive periodic acid–Schiff test given by some of these materials. The results of the present study suggest that the carbohydrate-protein complex in β- and γ-caseins may not contain freely reactive 1,2-glycol groups.

Filter paper chromatography was employed for the quantitative and qualitative analysis of the sugars present in whole casein (Warner), alcohol-extracted casein (Warner), α-casein, β-casein, and γ-casein. The whole casein preparation, which had been washed with water and then with alcohol and with ether to remove moisture, contained galactose, glucose, and mannose in the ratio of approximately 3.5:2.0:1. The nitrogen content was 15.05% (dry matter basis). It was considered possible that this preparation might contain an appreciable

TABLE 2

Sugar content of whole casein and of casein fractions[a]

	Series 1			Series 2		
	Galactose	Glucose	Mannose	Galactose	Glucose	Mannose
	(mg/g)					
Whole casein (Warner)	1.85(0.095,[b]5.1[c])	1.11(0.028,[b]2.5[c])	0.53(0.020,[b]3.8[c])	1.83(0.080,[b] 4.6[c])	1.10(0.028,[b]2.55[c])	0.53(0.014,[b]2.64[c])
Whole casein (alcohol-extracted)	1.34(0.025, 1.87)	0.00	0.56(0.028, 5.0)	1.34(0.025, 1.9)	0.00	0.56(0.020, 3.57)
α-Casein	0.74(0.045, 6.1)	0.00	0.47(0.028, 6.0)	0.75(0.078, 10.4)	0.00	0.49(0.035, 7.1)
β-Casein	0.51(0.040, 7.8)	0.00	0.00	0.53(0.032, 6.1)	0.00	0.00
γ-Casein	0.47(0.032, 6.8)	0.00	0.00	0.49(0.025, 5.1)	0.00	0.00

[a] Based on 15% nitrogen.
[b] Standard deviation.
[c] Coefficient of variation.

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amount of residual lactose. Accordingly, the preparation was subjected to Soxhlet extraction with 95% alcohol for 96 hr. The casein was then dried. The nitrogen content was 15.42% (dry matter basis). The extraction had reduced the galactose content by approximately 28%, but the mannose content was practically unchanged. Glucose, moreover, could no longer be detected by filter paper chromatography.

The simultaneous reduction of the galactose and glucose contents of whole casein by alcohol extraction could be explained by the removal of lactose contamination. However, if lactose were the only contaminating sugar removed by alcohol extraction, then the reduction in galactose content might be expected to be approximately equal to the reduction in glucose content. The fact that approximately twice as much glucose as galactose was removed by alcohol extraction suggests that glucose must have been present in the casein preparation in some other form as well as lactose.

It will be observed, from the data reported in Tables 1 and 2, that the sugar contents of α-, β-, and γ-casein do not account for the carbohydrate of the whole casein. This may point to a loss of carbohydrate-containing material during the preparation of the subfractions of the whole casein. The yield of α-, β-, and γ-casein in no instant accounted for 100% of the original casein, and it is possible that the material lost in preparation may have a high carbohydrate content. However, this point can be settled only by further research.

It is of interest to note that β- and γ-caseins contain only galactose, while α-casein contains galactose and mannose. It remains to be seen whether mannose is responsible for the fact that, of the casein fractions, only α-casein gives a positive periodic acid–Schiff test.

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A RAPID METHOD OF DETERMINING DISPERSIBILITY OF NONFAT DRY MILK SOLIDS

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SUMMARY

A mechanical method of determining dispersibility of nonfat dry milk using a Hobart Model 3C Kitchen Aid Mixer is described. A 5-sec. stirring time is used which gives better differentiation of grades than can be obtained by previously described procedures using longer stirring times. The mixture is screened and the amount of solids dispersed is determined by a hydrometric procedure developed for this purpose. The hydrometric procedure used was demonstrated to be as accurate as gravimetric determination of solids by oven drying. The approximate percentages of various types of nonfat dry milks dispersed using this procedure are: instant, 80%; ordinary spray-dried, 37%; and roller-dried, 0%. The differences in dispersibility observed between various brands of instant milks are large enough to be significant for grading purposes. The procedure, besides readily separating nonfat dry milk into dispersibility classes, has the feature of being very fast, the whole procedure requiring only 10 min.

The ease with which dry milk samples disperse in water is an important factor in evaluating their quality. Several methods (2, 10, 11) have been described for the objective determination of dispersibility, none of which has gained general acceptance.

Stone *et al.* (11) have described an objective method employing mechanical stirring with a Hobart Model 3C Kitchen Aid Mixer, followed by screening and determination of the amount of material which has gone into solution. Methods based on this procedure have recently been studied collaboratively under the supervision of the American Dry Milk Institute (2). The collaborative study indicated that some improvement in the methods is desirable.

One of the major problems in dispersibility measurements is the rapid and accurate determination of the dissolved solids. Two methods of determining total solids in milk are described by the A.O.A.C. (1). The first of these involves drying a weighed sample of milk to constant weight at 100° in an oven, whereas the second, described as an approximate method, uses the Quevenne lactometer readings and the per cent fat to calculate total solids. A number of firms now manufacture special equipment for rapid moisture determination by drying.

Considerable work has been done with determination of solids by the use of lactometers, and highly refined equations have been developed for the calculation of total solids from the specific gravity and the per cent fat (3, 4, 8, 12). Reinart and Nesbitt (7) compared some of these procedures with the Mojonnier gravimetric method and found they agreed fairly well. Madden *et al.* (5) also compared a variety of lactometric procedures with gravimetric results and obtained fair agreement.

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Michle *et al.* (6) compared the Mojonnier method, Cenco moisture balance, and the Karl Fischer titration, and concluded that the Mojonnier method was much superior. The A.D.M.I. collaborative study (2) on dispersibility methods included a comparison of the Mojonnier and the A.O.A.C. Method I. The Mojonnier method was found to give less satisfactory results in some cases.

The present paper describes a rapid method of determining dispersibility. It is based on the method of Stone *et al.* (11), with some important modifications which contribute to its simplicity and precision.

A. *Apparatus*

Mixer—Hobart Model 3C Kitchen Aid, equipped with standard agitator and automatic (mercury tilting) switch for starting agitator when the agitator is in the bowl.[1]

Sieves—8 in., 60-mesh and 100-mesh, together with collecting pan.

Graduated cylinders—250 ml.

Balance—Torsion or any balance which will weigh accurately to 0.1 g.

Hydrometer—Range 1.000–1.070 calibrated to 0.0005 subdivisions (Fisher).

Flasks, pipets, weighing dishes, stirring rods.

Timer—One which may be set to turn off stirring after a predetermined time.

Thermometer.

B. *Procedure*

The sieves are arranged with the 60-mesh on top of the 100-mesh and the collecting pan below. The stirrer is set at Speed 2 (approximately 77 r.p.m.) and hooked in the circuit with the timer, which is set to turn off after 5 sec.: 235 ml. of water at room temperature (20–30° C.) is poured into the mixing bowl, 35 g. of the milk powder to be tested is weighed out and carefully poured on the water in the mixing bowl. The agitator is immediately lowered into the mixing bowl, turned on and allowed to run 5 sec., at which time the clock will automatically stop the stirring.

The agitator is then removed, the liquid swirled once in the bowl, then poured into the sieves. The sieves may be shaken gently to speed filtration, which should be complete in 1 min. The liquid in the pan is then poured with the aid of a stirring rod into a 250-ml. graduated cylinder and water is added to the 250-ml. mark. The liquid is mixed thoroughly by pouring into a beaker and back again. Foam on the surface is drawn off carefully with a pipet attached to an aspirator. It is usually advisable to allow the sample to stand 5 min., to allow all foam to rise to the surface.

The hydrometer is placed in the liquid and allowed to bob up and down to come to equilibrium. The hydrometer is read to the graduation nearest the top of the raised area around the stem. The temperature of the milk is also recorded.

[1] The mention of specific instruments or trade names is made for the purpose of identification and does not imply any endorsement by the U. S. Government.

C. *Calculation of results*

The hydrometer should be checked for accuracy with water. If necessary, a correction may be added or subtracted to give the true density of the solution. The hydrometer reading (H) gives the specific gravity of the sample relative to water at 4° C., and must be divided by the density of water at the temperature of the sample to give the specific gravity (G) relative to water at this temperature.

To calculate the per cent solids, the formula for calculating total solids used in the A.O.A.C. Method II (lactometric) (1) was modified to

$$0.25\ (1{,}000)\ (G-1) + 0.3 = \%\ \text{solids}. \tag{1}$$

The amount of fat in nonfat dry milk is small and relatively constant and so may be neglected when this equation is used.

The hydrometer reading (H) times 250 gives the total weight of the solution. The number of grams of the powder which dissolved may be calculated by the formula

$$\text{Grams dissolved} = \frac{250\ \ (H)\ \ (\%\ \text{solids})}{100}. \tag{2}$$

The sample of dry milk may be assumed to contain 3% moisture unless the true moisture content is known. The actual amount of solids in a 35.0-g. sample then is 34.0 g. Formula (3):

$$\%\ \text{dissolved} = \frac{\text{weight dissolved} \times 100}{34.0} \tag{3}$$

or, combining Formulas (2) and (3),

$$\%\ \text{dissolved} = \frac{250\ \ (H)\ \ (\%\ \text{solids})}{34.0} \tag{4}$$

Illustration

A sample calculation to illustrate the use of the above formulas follows:

A. Calibration of hydrometer:

H_2O at 23° C.	
Hydrometer reading	0.9990
Density H_2O at 23°	0.9976
Approximate correction	−0.0014

B. Calculation of per cent dispersed (sample):

Hydrometer reading	1.0370
Correction (from above)	−0.0014
Corrected hydrometer reading (H)	1.0356
Temperature	24°
Density of water at 24°	0.9973 g/ml

$$\text{Specific gravity (G)} = \frac{1.0356}{0.9973} = 1.0385$$

From Formula (1)

$$\text{\% solids} = 0.25\ (1{,}000)\ (G-1) + 0.3$$
$$= 0.25\ (38.5) + 0.3$$
$$= 9.9\%$$

From Formula (4)

$$\text{\% dispersed} = \frac{250\ \ (H)\ \ (\text{\% solids})}{34.0}$$
$$= \frac{250\ (1.0356)\ (9.9)}{34.0}$$
$$= 75.5\%$$

RESULTS AND DISCUSSION

In preliminary experiments with a procedure based on that of Stone *et al.* (10), it was observed that a much better spread between samples was obtained if the stirring time was shortened from 10 to 5 sec. It appears that 5 sec. is insufficient time to control accurately. However, in practice this was no problem and good precision was invariably obtained, even when the stirring time was controlled manually. The temperature of the water ranged from 20–30° C., which had no noticeable effect on the rate of dispersion in this temperature range.

The greater spread between samples of various dispersibilities obtained by the shorter stirring time means that less precision is required to obtain meaningful results.

A specific gravity method for determining solids was adopted to speed up the procedure. Commercial lactometers are designed for use at lower temperatures than were convenient for our work; therefore, an ordinary hydrometer was used.

To calculate the per cent of dissolved solids, the formula from the A.O.A.C. Method II (lactometric) (1) was tested. The formula is:

$$0.25L + 1.2\ (\%\ \text{fat}) = \%\ \text{solids} \qquad (5)$$

The term (L) corresponds to 1,000 (G−1). One per cent fat was assumed to be present in the dry powder in calculating results using this equation. This will give a fat concentration near 0.1% in the reconstituted milk.

The accuracy of this method was checked by completely dissolving known amounts of different nonfat dry milk samples in water and comparing the actual amount dissolved with the results obtained from the formula. The percentages of solids calculated from Equation (5) were a little lower than would be expected from the amount dissolved.

Modification to:

$$\%\ \text{solids} = 0.25\ (1{,}000)\ (G-1) + 0.3 \qquad (1)$$

gave a good fit with the experimental data. Fat content of nonfat dry milk is assumed to be small and relatively constant and is allowed for in the constant.

Table 1 gives a comparison of the results obtained when a known amount of dry milk was dissolved in water and the resulting solution was analyzed for total solids by the specific gravity and the A.O.A.C. oven methods (1). We found it necessary to increase the drying time to 6–8 hr. by the oven method, to get constant weight and results comparable to the actual amount dissolved. In calculating the amount dissolved, allowance was made for the small amount of moisture in the dry powder. Moisture was determined by drying to constant weight at 100° C.; or, in the case of some instant samples, it was assumed to be 3%, as specified on the label.

TABLE 1

A comparison of the A.O.A.C. oven method with the specific gravity method of determining per cent solids in reconstituted nonfat milk

Sample	Known solids	Specific Gravity Method	A.O.A.C. Method
	(%)		
1	5.83	5.8	5.8
4	7.60	7.6	7.9
5	7.60	7.6	7.5
4	9.35	9.3	9.2
1	9.48	9.4	9.4
2	9.45	9.3	9.7
4	10.05	10.1	10.1
3	11.21	11.1	11.1
6	11.3	11.3	11.2
6	11.3	11.3	11.2
4	12.6	12.6	12.8
5	12.6	12.6	12.4

Our data show that the specific gravity method described is entirely satisfactory and invariably gives close agreement with the actual amount of dry milk dissolved. Somewhat better precision and accuracy were obtained from specific gravity measurements than from the A.O.A.C. oven method. In all but one case, the specific gravity method gave results agreeing within $\pm$ 0.1% of the actual amount dissolved. This is within the limit of error in reading the hydrometer, and is comparable to the accuracy obtained with other lactometric procedures (3, 4, 8, 12).

Table 2 shows the dispersibilities of various dry milk samples, including five different brands of instant milk powders, two ordinary spray-dried powders, and two roller-dried powders. Instant powders were about 80% dispersible by this procedure, whereas the ordinary spray-dried powders were only about 37% dispersible. Roller-dried samples were practically nondispersible by this procedure. The large differences in dispersibility observed allow the three types of dry milk to be readily differentiated. The reproducibility of duplicate determinations was sufficient to allow significant differences to be observed between individual samples of each type.

Many instant milk powders which appeared to disperse completely with no visible lumps formed a granular sludge in the bottom of the mixing bowl, which

TABLE 2
Dispersibility measurements

Sample	First determination	Second determination
	(%)	
Instant dry milks[a]		
Brand A	78.6	79.5
Brand B	80.5	79.0
Brand C	85.0	85.5
Brand D	79.0	77.7
Brand E	83.3	82.1
Ordinary spray-dried milks		
Sample 1	36.7	34.7
Sample 2	39.0	38.8
Roller-dried milks		
Sample 3	Approx. 0.0	Approx. 0.0
Sample 4	Approx. 0.0	Approx. 0.0

[a] Samples A–E were different brands of commercial instant dry milks purchased at a supermarket.

was collected on the screens. The amount of this undispersed material collected on the screens depends on the mesh of the screens and, therefore, if results are to be compared with those reported here, the size and mesh of screens specified must be rigidly followed. Some samples which gave visible lumps after stirring were completely free of this sludge. Therefore, visible lumps alone are not a completely reliable criterion of dispersibility.

Instant milk powders become lumpy and appear to lose their desirable dispersibility properties if not stored in tightly sealed containers or in a desiccator. This occurs in hot, humid, summer weather. This effect was not observed with ordinary spray-dried powders, which did not deteriorate noticeably under the same conditions. More study is needed on the stability of instant milk powders under various conditions.

These procedures are applicable only to nonfat dry milk. Problems of determining both total solids by specific gravity and dispersibility are much simplified, since the physical state of the fat in the milk is of no concern. The effects of fat are discussed by Heinemann *et al.* (3) and by Watson (12) for lactometric determination of solids, and by Sinnamon *et al.* (9) and Stone *et al.* (10) for dispersibility determinations.

ACKNOWLEDGMENTS

The ordinary spray-dried and roller-dried samples used in this study were obtained from the Chicago USDA, Agricultural Marketing Service Dairy Inspection Laboratory.

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GROWTH OF CERTAIN LIPOLYTIC MICROORGANISMS AT 4° C. AND THEIR INFLUENCE ON FREE FAT ACIDITY AND FLAVOR OF PASTEURIZED MILK

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SUMMARY

Twenty-five pure cultures of lipolytic microorganisms sometimes found in market-milk were inoculated into samples of high-quality pasteurized whole milk subsequently held at 4 ± 1° C. for a period of 12 days. At four-day intervals during the hold period the samples were examined for microbial content, free fat acidity, and developed flavors. Eighteen cultures grew reasonably well and brought about increases in free fat acidity. Seventeen of these produced a rancid flavor sometimes accompanied and/or followed by bitterness, whereas one caused the milk to be criticized as sour and unclean.

It has been known for many years that microbial lipolysis may contribute to the degradation of milk fat. But with the apparently increasing importance of psychrophilic microorganisms as a detriment to market milk quality, this phenomenon may be of more concern to the dairy industry today than in the past.

Palmer (10) and Rice and Markley (11) seem to have been among the first investigators to appreciate fully this type of lipolysis, as they were careful to incorporate preventatives in their experiments on the natural lipolytic activity in milk. More recent workers have published affirmative information. Anderson and Hardenbergh (2) reported the spoilage of refrigerated cream by an organism resembling *Achromobacter* or *Alcaligenes*. During this spoilage the only biochemical change worthy of note was the hydrolysis of fats, but there developed a bitter flavor accompanied by an irritating after-effect. Hammer and Collins (7) found that unsalted, raw cream butter held for as few as seven days at 6° C. frequently developed rancidity and contained large numbers of lipolytic bacteria. Davis (5) believed that bitterness in milk held at a low temperature might well be the result of butterfat degradation rather than the formation of "peptones." Babel (3) reported that the acidity of milk fat increased slowly in milk held at 4.4° C. and abrupt increases were generally accompanied by a marked increase in numbers of psychrophiles. Thomas (12) concluded that *Pseudomonas fluorescens* and *Pseudomonas fragi,* both known to grow very well at 5° C., may be the most common organisms responsible for rancidity occurring in butter. Such reports suggest a need for further information concerning the detrimental effect that lipolytic microorganisms may have on the quality of market milk held at refrigeration temperatures.

This study was made to determine the extent of fat hydrolysis and flavor development accompanying the growth in refrigerated, pasteurized whole milk of certain lipolytic microorganisms sometimes found in market milk.

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MATERIALS AND METHODS

Twenty-five pure cultures were used in this study. Twenty of these, No. 1 through 20, were isolated in the laboratory from samples of market milk held in refrigerated storage for various periods of time after purchase on the local market. These cultures were characterized and keyed to genus only, according to Breed *et al.* (4). Five cultures were obtained from the American Type Culture Collection (ATCC), Washington, D. C. Nineteen of the 20 laboratory isolates were distributed among three genera of bacteria, whereas Culture No. 16 was a yeast. The genera with their included cultures were *Pseudomonas,* No. 1 through 12; *Alcaligenes,* No. 13, 14, and 15; and *Achromobacter,* No. 17 through 20. The ATCC cultures were No. 21, *Alcaligenes viscolactis* (9036), No. 22, *Brevibacterium lipolyticum* (11367), No. 23, *Flavobacterium suaveolens* (958), No. 24, *Pseudomonas fluorescens* (11251), and No. 25, *Pseudomonas fragi* (4973). Each of the 25 cultures demonstrated the ability to hydrolyze butterfat within 15 days at 5° C., ten days at 10° C., and two days at 21° C. This was determined by streaking a loopful of a 24-hr. broth culture on Milk-Protein Hydrolysate (M-PH) agar containing 1% butterfat dyed with Nile Blue A, according to the method described by Knaysi (8), and incubating the streaked plates until positive results were obtained.

Stock cultures were maintained on M-PH agar slants in screw-cap culture tubes held at 4 ± 1° C. These were rejuvenated at monthly intervals by passing them through a 24-hr. incubation period in Tryptose Phosphate Agar (TPA) broth (1) at 21° C., then to fresh slants incubated 24 hr. at 21° C. before replacing them in 4 ± 1° C. storage. Working cultures were maintained in TPA broth in cotton-plugged culture tubes. These were transferred at seven- to ten-day intervals and held at 4 ± 1° C. after 24 hr. incubation at 21° C. Milk sample inocula were prepared by transferring a loopful of working culture to a tube of TPA broth and incubating it for 24 hr. at 21° C. A loopful of the 24-hr. culture was then transferred to 10 ml. of sterile skimmilk in a screw-cap culture tube which was held six days at 4 ± 1° C. before being used to inoculate a milk sample to be studied.

The milk samples used in this study were commercially pasteurized whole milk obtained from the University Creamery. All milk used originated in the same mixed-breed herd of cows. Fat content was standardized to between 3.5 and 4.0% before pasteurization. The average initial free fat acidity (FFA) as determined by the method of Frankel and Tarassuk (6) for all samples was 3.8. No milk was used having a flavor criticism on the day obtained, other than the cooked flavor. Within 15 min. after the end of the 30-min. hold period, quantities of milk of approximately one liter were aseptically removed from the pasteurizing vat and placed in sterile two-liter Erlenmeyer flasks packed in flake ice. These were quickly transferred to a room held at 4 to 6° C. The Erlenmeyer flasks were closed with rubber stoppers encased in butter parchment to prevent the adsorption of flavors from the stopper or the external atmosphere. Each series consisted of six samples of milk, one as a control and each of the other five inoculated with a pure culture of the lipolytic organism. The results of ten such series, including ten controls and duplicates each of 25 pure cultures, are reported.

The samples after cooling to 5 to 10° C. were inoculated with an amount of a six-day skimmilk culture estimated to give a Standard Plate Count (SPC) of not more than 30,000 per milliliter. However, with one trial of two cultures this number was exceeded. After inoculation, the samples were violently agitated and placed in refrigerated storage at 4 ± 1° C. Daily each sample was hand agitated until all visible traces of cream adhering to the flask were obliterated.

Within 30 min. after inoculation and on the fourth, eighth, and 12th day of refrigerated storage approximately 150 ml. were aseptically removed from each sample to a chilled, sterile 250-ml. Erlenmeyer flask which was then closed with a parchment-encased cork. These 150-ml. portions were removed immediately after hand-agitating the sample with 50 vertical strokes of about 2 ft. The portions were plated for both SPC and psychrophilic plate count (PPC) while still at storage temperature. The plate counts were made according to *Standard Methods for the Examination of Dairy Products* (1), using M-PH agar. Plates for SPC were incubated at 32° C. and those for PPC were incubated at 5° C. for seven days. After plating, the remainder of each 150-ml. portion was warmed to 20 ± 1° C. in a water bath. After sufficient hand-agitation to effect fat dispersion, duplicate 10-ml. volumes were pipetted into 60-ml. centrifuge tubes for determination of free fat acidity (6). The remaining milk was then examined organoleptically by an experienced milk judge, assisted at times by one or two others. All flavor criticisms as made by the judge were recorded, with no attempt to assign numerical scores to the samples.

RESULTS AND DISCUSSION

As indicated by the plate counts recorded in Table 1, 18 of the 25 cultures studied grew reasonably well in pasteurized whole milk held at 4 ± 1° C. Even though all cultures used in this study grew and hydrolyzed butterfat on an agar plate at 5° C., four in the genus *Achromobacter,* No. 17 through 20; one *Alcaligenes,* No. 13; *Alcaligenes viscolactis* (9036), No. 21; and *Flavobacterium suaveolens* (958), No. 23 made little or no growth in the pasteurized milk during the 12-day period. Possibly this was due to the relative size of the inoculum used on the agar plate and in the pasteurized milk sample.

As indicated in Table 1, Cultures 2, 10, and 11 produced high counts on the psychrophilic plates, but these cultures were unable to develop countable colonies at 32° C. as shown by their Standard Plate Counts. Also, with many of the other cultures higher counts were obtained at 5 than at 32° C. Nelson and Baker (9) found that many bacteria from refrigerated milk failed to develop countable colonies at 32° C.

The very low average SPC maintained by the ten control samples during the hold period is of some interest. Also, the apparent absence of psychrophilic microorganisms in the control samples aseptically removed from the pasteurizing vat agrees with the reports of several workers, as reviewed by Thomas (12), that efficient pasteurization destroys those psychrophilic types commonly found in milk. During preliminary trials of this study several attempts were made to utilize commercially pasteurized milk available to this Station from different

TABLE 1

Standard plate counts (SPC) and psychrophilic plate counts (PPC) on samples of pasteurized whole milk inoculated with pure cultures of lipolytic microorganisms and held 12 days at 4± 1° C. Each (except for the control) is the average of two trials per culture

	Days of storage							
	0[a]		4		8		12	
Culture	SPC	PPC	SPC	PPC	SPC	PPC	SPC	PPC
	(plate counts/ml)							
Control[b]	320.0	<10	330.0	<10	320.0	<10	310.0	<10
1	4.9T[c]	47.0T	42.0M[d]	85.0M	82.0M	310.0M	180.0M	340.0M
2	390.0	990.0T	1.9T	33.0M	3.7T	110.0M	2.3T	170.0M
3	6.4T	34.0T	21.0M	59.0M	57.0M	160.0M	100.0M	260.0M
4	2.7T	44.0T	6.1M	78.0M	56.0M	150.0M	130.0M	150.0M
5	6.7T	6.3T	6.5M	13.0M	450.0M	490.0M	490.0M	490.0M
6	9.8T	35.0T	1.2M	16.0M	32.0M	130.0M	150.0M	290.0M
7	15.0T	18.0T	82.0M	14.0M	230.0M	310.0M	600.0M	360.0M
8	7.8T	9.8T	260.0T	530.0T	24.0M	71.0M	37.0M	88.0M
9	21.0T	45.0T	2.1M	9.1M	28.0M	23.0M	190.0M	650.0M
10	360.0	240.0T	650.0	1.4M	550.0	180.0M	580.0	360.0M
11	320.0	230.0T	320.0	1.9M	680.0	160.0M	590.0	570.0M
12	8.8T	9.0T	240.0T	550.0T	48.0M	160.0M	350.0M	130.0M
13	9.3T	700.0	12.0T	230.0	33.0T	790.0	140.0T	850.0
14	83.0T	70.0T	710.0T	900.0T	58.0M	68.0M	460.0M	420.0M
15	56.0T	24.0T	480.0T	390.0T	150.0M	130.0M	380.0M	340.0M
16	2.0T	1.5T	29.0T	31.0T	1.7M	960.0T	31.0M	6.4M
17	15.0T	17.0T	670.0	560.0	2.0T	630.0	290.0	80.0
18	4.3T	2.3T	2.4T	720.0	580.0	460.0	55.0T	30.0
19	14.0T	25.0T	1.1T	960.0	830.0	380.0	240.0	130.0
20	5.6T	3.6T	370.0	320.0	1.9T	230.0	200.0	60.0
21	1.2T	260.0	900.0	1.9T	130.0	280.0	210.0	75.0
22	6.8T	4.8T	6.4M	6.3M	290.0M	270.0M	1100.0M	880.0M
23	250.0	130.0	380.0	3.3T	210.0	280.0	360.0	150.0
24	7.7T	1.1T	100.0T	580.0T	12.0M	4.2M	21.0M	23.0M
25	13.0T	98.0T	1.9M	10.0M	17.0M	53.0M	55.0M	230.0M

[a] Within 1 hr. after inoculation.
[b] Average of ten trials.
[c] Thousands.
[d] Millions.

sources. All trials using milk not aseptically removed from the pasteurizer failed. These failures were attributed to post-pasteurization contamination of the milk with psychrophilic microorganisms, which precluded its use as a growth medium in the pure culture study of similar types. In most of the unsuccessful trials the uninoculated control samples were spoiled by the eighth day of the desired 12-day hold period at 4 ± 1° C.

A comparison of the data in Tables 1 and 2 shows that with these cultures good growth in milk at 4 ± 1° C. was accompanied by an increase in the FFA of that milk. Such increases are attributable only to the lipolytic action of the microorganisms, as no similar increases occurred in the control samples or in samples having little or no growth.

Rancidity and bitterness were the predominant flavor defects (Table 3) developing in inoculated milk samples having good growth with increased FFA, while the flavor of the controls and low-growth samples remained unchanged. Several times during the study rancidity was detected after a very slight increase in FFA and on one occasion as low as 0.9 for one trial with Culture No. 7.

TABLE 2

Increases in the free fat acidity of pasteurized whole milk inoculated with pure cultures of lipolytic microorganisms and held 12 days at 4 ± 1° C.[a]

Culture	Days of storage		
	4	8	12
Controls[b]	0.0	0.0	0.0
1	10.9	45.4	81.3
2	19.0	54.1	76.7
3	6.7	38.6	65.9
4	7.5	39.7	60.2
5	0.0	11.4	32.9
6	1.2	19.8	45.8
7	0.8	3.3	3.3
8	0.1	2.0	6.2
9	0.1	2.2	6.8
10	0.3	3.0	8.1
11	0.2	3.1	9.1
12	0.3	2.2	11.7
13	0.0	0.0	0.0
14	0.0	0.6	2.4
15	0.0	0.7	4.9
16	0.0	0.6	1.6
17	0.0	0.0	0.0
18	0.0	0.0	0.0
19	0.0	0.0	0.0
20	0.0	0.0	0.0
21	0.0	0.0	0.0
22	0.0	1.8	8.5
23	0.0	0.0	0.0
24	0.0	3.6	9.0
25	5.4	46.6	83.4

[a] Each (except for the control) is the average of two trials per culture expressed to the nearest tenth.

[b] Average of ten trials.

TABLE 3

Summary of typical flavor changes occurring in pasteurized whole milk held 12 days at 4 ± 1° C. after inoculation with pure cultures of lipolytic microorganisms

Culture group[a]	Days of storage		
Pseudomonas—I Cultures 1, 2, 3 and 4.	Slightly rancid to rancid	Rancid odor and/or flavor. Sometimes bitter	Rancid odor and/or flavor. Usually very bitter
Pseudomonas—II Cultures 5 and 6	None detectable	Rancid odor and/or flavor. Sometimes bitter	Rancid odor and/or flavor. Usually very bitter
Pseudomonas—III Cultures 7, 8, 9, 10, 11 and 12.	Usually little or no change. Sometimes slightly rancid	Usually slightly rancid. Rarely bitter	Rancid odor and/or flavor. Usually very bitter
Alcaligenes Cultures 13, 14 and 15.	None detectable	Sometimes slightly rancid	Usually rancid
Yeast (unidentified) Culture 16.	None detectable	None detectable	Slightly rancid
Achromobacter Cultures 17, 18, 19 and 20.	None detectable	None detectable	None detectable

[a] Representative of two trials for each of 20 cultures.

Seventeen cultures produced a rancid flavor in milk during the storage period. Six of these developed detectable rancidity by the fourth day, eight others by the eighth day, and the remaining three by the 12th day. With ten cultures, bitterness also developed, but in no instance did it precede rancidity. In only a few trials was bitterness noticeable at the same time that rancidity was first detected.

Brevibacterium lipolyticum (11367), No. 22 was the only organism studied which grew well and brought about measurable increases in the FFA of the milk, yet produced neither rancidity nor bitterness. Its average increase in FFA of 8.5 after 12 days was considerably higher than many of the other cultures where rancidity was detected. Yet in both trials the sample inoculated with this organism was criticized after 12 days of storage as being sour and unclean. This was not expected, since the organism is not characterized as being able to ferment lactose (4). However, in this laboratory it produced a gassy, acid-appearing curd in litmus milk within five days at 21° C.

The data on flavor development presented in Table 3 are a summary compiled from the organoleptic evaluations of the milk samples on the days they were analyzed for microbial content and FFA. It represents the results of two trials for each of the 20 cultures of microorganisms isolated in the laboratory. The cultures are grouped generically, with those in the genus *Pseudomonas* separated into three groups with members of comparable lipolytic ability. This grouping is based on the two-trial, 12th-day average increase in FFA produced by each culture. On this basis, *Ps. fragi* (4973) falls into group *Pseudomonas*—I and *Ps. fluorescens* (11251) into *Pseudomonas*—III.

In general, according to the results of this study, flavor development by pure cultures of lipolytic microorganisms growing well in milk at $4 \pm 1°$ C. follows a fairly definite pattern. The milk first loses its original flavor to become slightly rancid. This is followed by a more distant rancidity, which may or may not be accompanied by bitterness. Usually, if microbial degradation of the milk constituents is allowed to continue, the milk develops an extreme bitterness, which masks rancidity except as detected by the sense of smell. It is doubtful that the bitterness in the latter stage is solely the result of fat hydrolysis as frequently by this time changes in the protein have occurred.

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ULTRA-HIGH-TEMPERATURE SHORT-TIME EXPERIMENTAL STUDIES ON FLUID MILK PRODUCTS. EXPERIMENTAL EQUIPMENT[1]

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SUMMARY

Two heaters have been used to experimentally sterilize small quantities of fluid milk products. The Mallory tubular heater has a capacity of 200 gal/hr and a product flow-rate of 23 ft/sec, but it can be increased to 300 gal/hr. Sections of this heater can be used either as pre-heating, heating or holding tubes for a calculated time of 2.08 sec. each, and they are accurate and efficient, due to the high ratio of length to diameter (2,296:1). The products can be homogenized either before or after sterilization, or both, and over a range of temperatures and pressures.

The DeLaval plate heater has a heating, a cooling, and two regenerating sections which make it possible to homogenize the product at different temperatures. The plates can withstand pressures up to 60 lb/in^2, are 0.043-in. thick with a corrugated profile, and have 1.51 ft.^{2} each of surface. The K factors (B.t.u/hr/ft^2/° F.) for the heating, regenerating, and cooling sections are 700, 300, and 500, respectively. Vacuum- and pressure-steam can be used, depending on the desired temperatures. Two temperature controls are used, one with a vacuum pump in the condensate side of the heating system for about 220° F. and lower, and the other one with a steam trap, to maintain temperatures of about 300° F.

An apparatus was constructed in which milk products can be put aseptically, in sterile glass bottles and in sterile metal cans, either in a normal atmosphere or in one of an inert gas. Detailed dimensions are given.

Ultra-high-temperature, short-time heating of fluid milk products has revealed new research frontiers that need to be investigated for the effects of heat on the palatability, physico-chemical properties, and keeping qualities of these products. In preparing for such studies at the Illinois Agricultural Experiment Station, equipment of small capacity was obtained, altered, and arranged so that experiments could be conducted economically and under controlled conditions. This equipment included a Mallory small-tube heater with an attached homogenizer, a DeLaval plate heater with regenerating and cooling sections, a Manton-Gaulin homogenizer, and an apparatus in which milk can be bottled or canned aseptically, including other specially made utensils. This equipment, and procedures for preparing it for processing sterile fluid milk products, will be described.

Mallory small-tube heater. This heater has been used for experimental purposes (2, 3, 7). More recently (4), it was equipped with a two-stage homogenizer head, which is mounted on an adjustable stand and may be attached at six different positions on the cooling sections, depending on the desired homogenizing temperatures. Since the tubes in the heating and cooling sections will withstand pressures up to about 6,000 p.s.i.g., it is possible to experimentally homogenize milk products at as much as 4,000 to 4,500 p.s.i.g., the difference of

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1,500 to 2,000 p.s.i.g. being the pressure required to pump these products through 143.5 ft. of stainless steel tubing 0.25 in. in diameter in the heating section and the same amount of tubing in the cooling section.[2] It required about 2,000 p.s.i.g. to pump concentrated milk (11.12% fat and 26.73% solids-not-fat) through the heating and cooling sections. A homogenizer, equipped with a 10-h.p. motor, served as the pump. Thus, it was possible, experimentally, to homogenize the same product either before or after sterilization or both before and after sterilization, and over a range of different temperatures and pressures. Furthermore, the product may be heated in Section A (Figure 1) and Sections B and C may

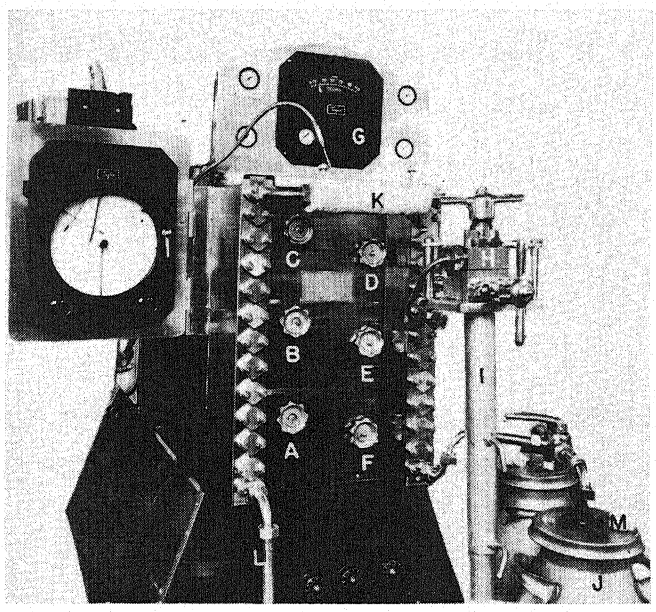


FIG. 1. Mallory heater. A, B, C. Valves for heating sections. D, E, F. Valves for cooling section. G. Steam pressure regulator. H. Two-stage homogenizer. I. Support. J. Sterile can with vent and female connection. K. Flow-line to cooler, containing temperature-recording bulb. L. Feed line from homogenizer-pump. M. Vent on can.

be used for holding it for 4.16 sec.; or Section B may be used for heating and Section C for holding the product for 2.08 sec.; or Section B may be used for heating with only a very short or no calculated holding time, depending on the temperature desired and the product being processed, as the rate of heating depends on the steam pressure and the composition and viscosity of the product. It is possible also to use Section A for preheating milk before sterilization by equipping it with temperature controls, then using Sections B and C for heating and/or holding, respectively. This has been done by manually controlling the flow of

[2] When the homogenizer (H, Figure 1) was installed, 12 ft. of tubes in the cooling section section had to be closed. These closed tubes are about equal to the tubes leading to, to the space within, and to the tubes leading from the homogenizer.

steam into Section A. Sections A, B, and C are equipped with positive individual valves that shut off the steam. Finally, it is possible also to use either one or two or all three Sections D, E, and F for holding the product at a specified temperature by shutting off the coolant, each one holding the product for 2.08 sec. If all of these sections are used as a holding tube, then it would be necessary to install an auxiliary unit to cool the products.

Each tubular section is 47.83 ft. (573.9 in.) long and 0.25 in. wide with eight return bends, giving a length-to-diameter ratio of 2,296 : 1. This ratio is more than five times that stated by Walzholz (9), who described a holding tube in which the ratio of the fastest to the slowest moving particle was 1.25 : 1, the ideal ratio being 1 : 1. Therefore, it can be assumed that each section in the Mallory heater is an efficient holding tube. If the volume were increased to 300 gal. per hour, which is possible, then the holding and heating times would be decreased, proportionately, by 50%.

Since the homogenizer is attached to one of the cooling sections, the product is under pressure through all of the heating sections. For this reason, the fluid milk product can not undergo more than thermal expansion. This retards the adsorption (burn-on) of milk solids on the inside of the tubes. Without this equalization of pressure throughout the heating section, there would be faster adsorption of milk solids, which would show an increased pressure on the gauge of the homogenizer-pump.

The mean calculated velocity of the product at 200 gal/hr was 23 ft/sec through the heating and cooling sections of the Mallory heater, the total time being 12.48 sec. The minimum and maximum velocities at a given instant were not determined. They would be a function of the intervals between thrusts of the homogenizer which served as the pump. At 300 gal/hr, the mean velocity would be 34.5 ft/sec.

The Mallory heater, the attached homogenizer, and three-way valve assembly with male connections, were sterilized by pumping distilled water at 300 to 310° F. through the heating and cooling sections for 10 to 12 min. to sterilize these parts; the outside surfaces of these parts have varying exposures to ambient temperatures.

Gaskets on the headers (return bends) of the heating and cooling sections had to be replaced occasionally. These gaskets were kept in germicidal solutions for 12 to 17 hr. before they were installed. Sometimes during pasteurization or sterilization, small amounts of the milk product would seep past some of the gaskets, due to not tightening the nuts on the headers sufficiently and uniformly or due to excessive amounts of air in the product. When this occurred, the gaskets were replaced because they became a source of post-pasteurization or post-sterilization contamination.

DeLaval plate heater. The plate heat exchanger is shown in Figure 2. It consists of a heating section, two regenerating sections, and a cooling section. Split regenerating sections were used to make homogenization possible at temperatures other than the final heating or final cold regeneration when the product was

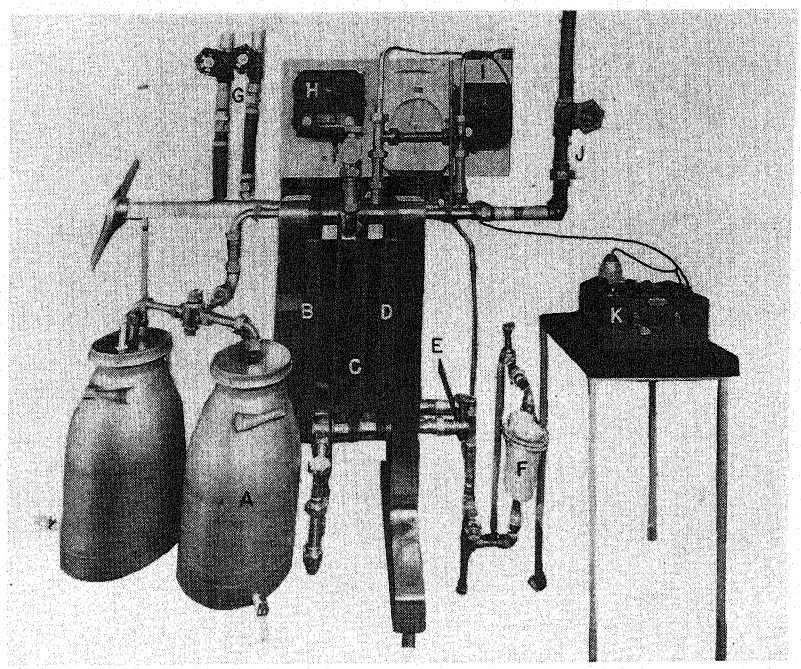


FIG. 2. DeLaval plate heater. A. Sterile can. B. Cooler. C. Regenerator. D. Heater. E. Vacuum steam line. F. High-pressure steam trap. G. Lines for cooling water. H. Steam pressure regulator for 220° F. and lower. I. Steam pressure regulator for 220 to 300° F. J. High-pressure steam line. K. Temperature potentiometer.

homogenized after heating, or final hot regeneration when homogenizing before heating. It is sterilized in the same way as the Mallory heater.

The plates were of the high-pressure type, 0.043 in. in thickness, with a corrugated profile for rigidity and to provide turbulence. The projected plate area was 1.29 sq. ft. and the actual area was 1.51 sq. ft. The distance between the plates was 0.032 in. and the volume of water held between two plates was 0.9 lb. Near their periphery, the plates had a bridging type recess which served as a seat for a synthetic rubber gasket. The maximum working pressure was 60 lb/sq in.

Vacuum-steam and pressure-steam were used as the heating media, depending on the desired temperatures. Two temperature control systems were required: one was operated with a vacuum pump on the condensate side of the heating system and was used at the lower temperatures, and the other required a steam trap to maintain the desired positive pressure on the heating side and was used at temperatures above 220° F.

Typical K factors (B.t.u/hr/ft^2/° F.) were found to be 700 for the heater, 300 for the regenerator, and 500 for the cooler. These values are applicable for milk heated to 200 to 300° F., using 75% regeneration and cooling to 38 to 40° F. The flexibility of the unit is illustrated in the normal rates of flow of 900 to 2,500 lb/hr/stream. It was usually operated at a capacity of 1,720 lb/hr for milk and 1,600 lb/hr for ice cream mix containing 37 to 38% of total solids.

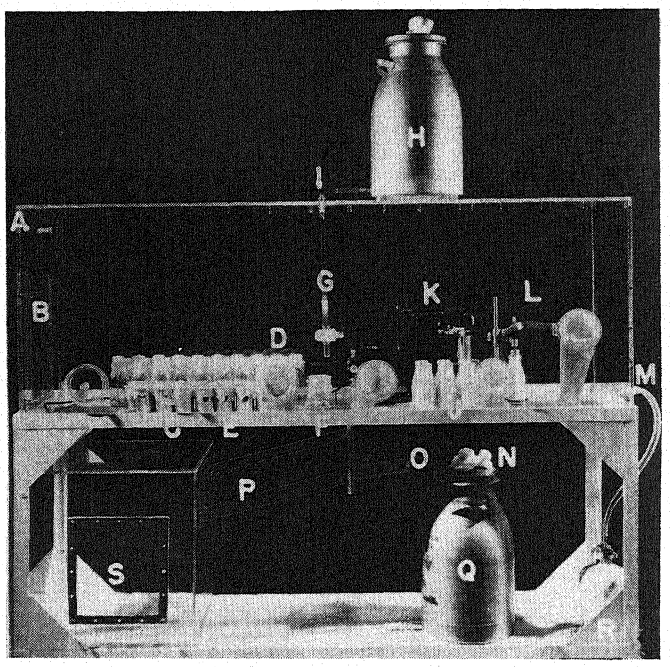


FIG. 3. Aseptic bottling and canning apparatus. A. Vent where germicidal solutions are sprayed into chamber. B. Opening through which equipment is placed in chamber. C. Attachments for rubber gloves. D. Glass bottles. E. Metal cans. F. Caps for bottles. G. Milk dispenser. H. Can for milk product. I. Connection to can. J. Filled bottles. K. Can sealer. L. Bottle capper. M. Air and/or gas line. N. Vent tube on can. O. Female fitting on can. P. Chute. Q. Sterile can. R. Filter. S. Compartment for holding filled cans.

Containers for collecting milk from the heaters. Ten-gallon cans, made of stainless steel, were used to collect milk from the cooling section of the heaters (Figures 1, 2). A gum-rubber tube about 3 in. long was attached to the outlet of each can. (I, Figure 3.) The end of this tubing was wrapped with parchment, which overlapped about 1.5 in., then it was fastened with a rubber band at the base and left open during sterilization. A layer of absorbent cotton, 2 in. wide and about 0.5 in. thick, was inserted between the outer and inner rim of the cover which was placed loosely on the can. The intake tube (O, Figure 3) with a sanitary connection was covered with cotton, then with parchment, and fastened with a rubber band. The vent (N, Figure 3) was also covered with cotton, which was fastened with a rubber band. The 10-gal. cans, with their covers loosely attached, were sterilized in the autoclave at 248° F. at 15 or 16 p.s.i.g. for 50 min. Immediately after, they were sterilized, the covers were securely fastened with wire to the handles of the can, and the parchment on the outlet tube was folded back 180° and fastened with a rubber band. When the cans had cooled, the rubber tube was closed with a pinch-cock.

Aseptic bottling and canning apparatus. Attempts were made to fill bottles with fluid milk products directly from the cooling section of the Mallory heater. Unfortunately, the bottles could not be filled uniformly and microorganisms in the

air infected the milk products. Therefore, it was necessary to construct an apparatus in which the milk products could be put in glass bottles or metal cans, aseptically. This apparatus was made of lucite, the joints of which were held together with screws 1.5 in. long and, in addition, these joints were sealed with lucite glue. This glue was made by dissolving 10 to 12 g. of finely ground lucite in 100 ml. of methylene dichloride, which flowed between the joints before the screws were tightened. This aseptic apparatus had a trap door, connected to a chute, through which cans of milk rolled to a lower compartment as soon as they were filled. The construction of parts, dimensions, and location of the different openings are shown in Figure 4. This was an improvement over the apparatus described previously (3).

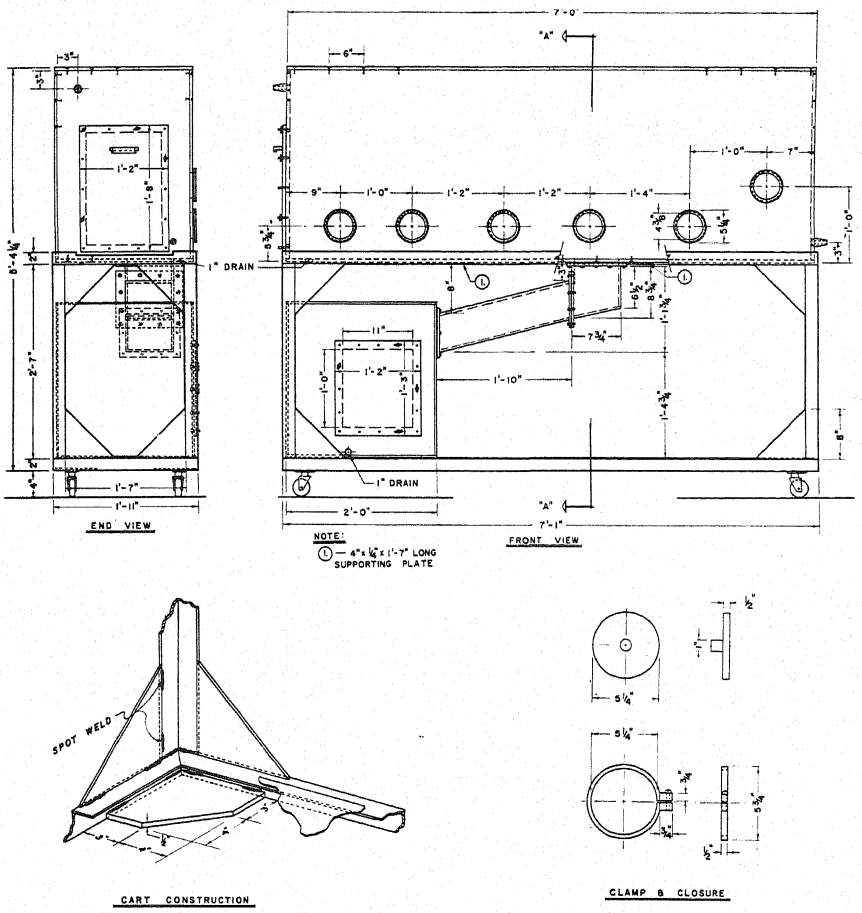


FIG. 4. Showing construction of aseptic bottling and canning apparatus with cart.

The bottling apparatus was supported on a cart, the frame of which was made from 2-in., 90° angle iron, 0.25 in. thick and reinforced at the corners with welded iron plate of the same thickness. This cart was mounted on four swivel-

type, rubber-covered wheels, 3 in. in diameter, which could be locked in place. A platform was provided for utensils and accessories. The entire apparatus could be moved into an air-cooled room.

Twenty gallons of a fluid milk product could be bottled or canned in one operation in containers of various sizes. These containers could be filled in an inert atmosphere by displacing the air with carbon dioxide or nitrogen gas and maintaining a slight positive pressure with these gases. It was necessary to sterilize the bottling and canning apparatus, the bottle capper, the can sealer, and the rubber gloves with germicidal solutions. The rubber gloves were dipped in solutions of either 500 p.p.m. of chlorine, quaternary ammonium, or hydrogen peroxide, and were attached with clamps to openings provided for them (C, Figure 3). Next the apparatus was sprayed through Opening A for about 1 min. with one of the germicidal solutions with an air-pressurized sprayer. The sterilized bottles and caps were placed inside the apparatus. An aluminum rod, the end of which was bent in a half circle, was also placed in the chamber and it was used to move empty and filled bottles or cans. The milk dispensing assembly was put in the operating position and the apparatus was again sprayed with the germicidal solution for about 30 sec. The filtering assembly was connected to a compressed air line and air was passed through the apparatus to remove the excess germicide. The door (B, Figure 3) was left partially open during this aeration. Schulz (6) has described a similar apparatus for bottling milk aseptically.

Milk dispensing assembly. The milk product in the 10-gal. can flowed through a glass tube, 15 mm. inside diameter, and into the asepticized apparatus, where it was put in glass bottles or in metal cans. This tube was fitted with 19/38 tapered, ground glass joints at its upper end and the female part had a tube 3 in. long, bent at 90°, connected to the 10-gal. can on top of the apparatus (I, Figure 3). A glass stop-cock was connected with 19/38 tapered joints to the lower end of the tube (G, Figure 3). Before sterilization, and to protect from contamination afterwards, parchment was rolled around and about 1.5 in. beyond the upper end of the tube and also at the end of the stop-cock. After sterilization, the parchment at both ends was folded back 180° and fastened with a rubber band.

Because of greater durability, the glass dispensing assembly was replaced with a stainless steel tube of the same inside diameter as the glass. It was bent 90° at the top to connect with the 10-gal. can and a glass tube was attached with a rubber tube at the lower end. The flow of fluid milk products into containers was regulated with a pinch-cock.

Milk containers. Glass bottles of pint and quart capacity were used. They had a specially made rim, 38 mm. inside and 42 mm. outside diameter. In some experiments, beverage bottles with opening 18 mm. inside and 26 mm. outside diameter and of clear, brown, and ruby glass were used. They were closed with friction crown caps. Metal cans of various sizes also were used.

The closures of the glass bottles and of the metal cans were covered with parchment, held in place with rubber bands. The metal caps for the bottles

and cans were placed in beakers covered loosely with parchment. They were sterilized for 40 min. at 15 to 16 p.s.i.g., then the parchments on the beakers were fastened securely with rubber bands as they were removed from the autoclave.

Bottle capper and can sealer. The stainless steel bottle capper, manually operated, was made in the University machine shop. It closed and crimped tightly the metal crown caps on beverage bottles of different sizes. It was used also to press the friction-type metal caps on to the closures of the milk bottles. The capper was fastened with bolts to the floor of the aseptic apparatus (L, Figure 3).

A can sealer operated by an electric motor was used to seal covers onto the metal cans.[3] It was not fastened permanently in the aseptic apparatus (K, Figure 3). There was room for both the bottle capper and the can sealer in the aseptic apparatus.

Air and gas filter. The air and gas filter for the aseptic bottling unit was made from a four-liter dispensing jar, filled with cotton and equipped with a clamp to hold the rubber stopper. This jar was connected to the air or to inert gas supplies and to the aseptic apparatus with plastic tubing, 20 mm. inside diameter. The two openings in the jar were plugged with cotton and the filtering assembly was sterilized in the autoclave for 50 min. A pressure of 30 p.s.i.g. was used to force air or inert gases through the filter and into the aseptic apparatus. The plastic tubing was sterilized with hydrogen peroxide. Several variations of the filter were used before this one was adopted (R, Figure 3).

The term aseptic is used to indicate that air-borne microorganisms were eliminated as sources of contamination. The vegetative organisms were destroyed by the chlorine, quaternary ammonium, or hydrogen peroxide germicides, but the spores probably remained dormant on the inner surfaces of the apparatus. However, hydrogen peroxide will destroy certain species of spores (5), though studies have been limited to a few species in milk with varying concentrations of the germicide.

To determine if it were possible to prevent air contamination in this apparatus, about 20 qt. of nutrient broth were poured into the 10-gal. can and sterilized in the autoclave, cooled, and bottled in the asepticized apparatus. Some bottles were filled completely, others to varying degrees of fullness. They were incubated at 70 ± 0.5° F. for 76 hr., then at 100 ± 2° F. for 97 hr. The broth remained clear, indicating no visible growth. Twelve bottles were selected at random and samples of broth from each one were plated by Standard Methods (1). No growth was observed on the Petri plates. In the same way, about 20 qt. of milk were sterilized, bottled, stored, sampled at random, and plated. No bacterial growth was observed. Removing the closures from the bottles of broth and milk, and exposing them to the air, resulted in the growth of air-borne microorganisms. Repeated trials have shown that sterile milk can be put aseptically

[3] Automatic Canning Devices, Inc., Manitowoc, Wisconsin, made this apparatus.

into glass bottles and into metal cans. Research results obtained with this equipment have been published (4) and other results will be forthcoming.

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SUMMER FEEDING OF DAIRY CATTLE. A REVIEW[1,2]

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SUMMARY

Income for dairy farmers during the summer depends to a considerable extent on more pasture herbage production in hot-dry weather. The results of recent studies indicate that dairy cattle fed an adequate ration, and allowed to graze nights and days with shade during the days, are far less affected by high environmental temperatures than previously supposed. Increased quality and quantity of forage may be enhanced by: (a) growing crops more resistant to heat and drought, (b) maintaining more moisture in the soil by less defoliation of plants which give shade to the soil, (c) irrigation, and (d) fertilization of the soil (especially with nitrogen, phosphorus, and potassium). The problems involved in using animal production (growth, milk yields) in pasture evaluation were pointed out. Indirect methods of pasture evaluation using internal and external indicators were described. A combination of internal and external indicators makes possible the estimation not only of the chemical composition but also of the coefficient of digestibility and dry matter intake of pasture. Dry matter intake from pasture is variable, but usually is about 30 to 35 lb. per day for cows weighing 1,200 lb. Grazed pasture herbage is higher in crude protein and lower in crude fiber and higher in digestibility than comparable hand-fed herbage. The requirements of grazing cattle are about the same as in barn-feeding, with the exception of digestible energy. Grazing cattle require from 1 to 3 lb. of TDN per cow per day more than those barn-feeding. Losses of pasture herbage from trampling, when properly managed, are about 10 to 15%. These losses are much higher with tall pastures. Temporary pastures, such as Sudan grass, are needed in many areas to supplement perennial pastures during the summer. The success of different systems of pasture utilization hinges on proper management (proper stocking rate, use of high-producing cows). Rotational and strip-grazing systems aid in using intensive pastures. Soilage may be used to advantage on farms where good pasture land is not conveniently located. Where soilage and stored feed may be used to advantage was pointed out. The fermentation vat of calves fed feeds other than milk alone is sufficiently developed at 2 to 4 mo. of age to handle good pasture, but the calves will starve on poor pasture. Certain pasture forages, such as Sudan grass, may contain considerable hydrocyanic acid. When properly managed, however, the danger from prussic acid poisoning is not great. Although the cause of pasture bloat is not known, recent results indicate that the administration of penicillin prevents bloat for a short time. Also, vegetable oils sprayed on "bloaty" pastures, added to the drinking water or to the feed, reduce the incidence of bloat. Milk produced by cows on pasture is more resistant to oxidized flavor than when winter feeds are fed. Pasture feed flavors may be avoided by removing cows from pasture 3 to 5 hr. prior to milking.

One of the most important problems facing the dairy farmer in many areas of the world is the decline of milk production during the summer months. The critical period between the abundant pasture supply in spring and the revival in the fall is associated with high temperatures, low humidity, and insufficient rainfall. Profitable dairy production is based on the ability to utilize known information about management of both cattle and forage crops. This is especially important in overcoming the decrease in pasture production, which results in a decline in milk yields, and the increase in feed costs due to the feeding of harvested herbage and concentrates.

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[2] Reprints available for 90 days after publication of this paper. For prices see page 1434 in this Journal for August, 1959.

This review is primarily concerned with the economics of pasture feeding, the effects of summer climate on milk production, pasture crop production, changes in nutritive value of forage crops, methods of measuring pasture production, the uses of pasture, and the relation of pasture to disease and milk quality. No attempt will be made in this review to cover all of the areas of summer pasture production, such as the breeding of forage plants resistant to drought and high temperatures, or the value of different species of permanent and temporary pasture crops. The role of the fermentation vat in herbage utilization, as well as plant metabolism dealing with reduced digestibility with maturity, will not be discussed in the review. References to many reviews are included in lieu of a more complete literature coverage.

ECONOMIC IMPORTANCE OF PASTURE

The economics of pasture feeding compared with feeding harvested crops appears to justify the use of land for pasture similar to that now used for cultivated crops (303). Unfortunately, the data for the economics of pasture usually include the entire pasture season, although it is well recognized that pasture herbage is worth more during the summer months, in most areas, than that produced in the spring or fall.

The data relative to the cost of producing 100 lb. of TDN from various crops grown in 16 states are shown in Table 1 (312). Ahlgren *et al.* (4) concluded that pastures offer the livestock producer his cheapest and most economical source of feed. When cows were pastured from mid-May to mid-October, there was a reduction of 46% in the amount of grain fed, 86% in roughage fed other than pasture, and 31% less labor used daily in caring for the cow (75). The average reduction in feed and labor costs per cow per day, after charging the cost for pasture, was 48% below that for the winter-feeding period.

Studies in New Jersey (survey) showed that pasture was the cheapest source of TDN (49). The costs per 100 lb. of TDN from different sources were: pasture, \$1.26; hay, \$1.74; silage, \$2.07, and concentrate feed, \$4.23. The studies showed that many farmers were getting only about 500 lb. of TDN from each acre of pasture. Better-managed pastures, however, yielded 1,500 lb. of TDN per acre.

TABLE 1

Cost of producing 100 lb. of total digestible nutrients from various crops (312)

Crop	TDN/acre	Cost/100 lb.
	(lb.)	*(\$)*
Oats	932	2.02
Wheat	1,146	1.88
Barley	1,217	1.70
Corn silage	2,320	1.54
Corn grain	1,778	1.38
Timothy hay	1,257	1.21
Clover and timothy hay	1,347	1.15
Soybeans	1,725	1.06
Red clover hay	1,622	0.97
Alfalfa hay	2,522	0.83
Pasture	.....	0.64

The unit cost of producing milk on farms near Chicago was considerably less in summer than in winter (305). The cost of producing 100 lb. of milk in summer (May-September) was $1.41, vs. $1.82 in winter (October-April). The cost of producing milk was lowest during June and July and highest in January, February, and March. In January, the feed cost for producing 100 lb. of milk was nearly twice as much as in June, when the cows were on pasture. Similar results were reported for the St. Louis milk shed (306).

According to an Indiana study, pastures did not produce as much feed per acre as when harvested crops were grown on the land (315). When the expenses for labor and machinery for planting, tilling, and harvesting crops are considered, the net return from pasture may exceed the net return from harvested crops on the same land. Wright and Hodge (313), in a study of milk production costs in southeastern Michigan, concluded that pasture at $21 per cow for the season provided nutrients at about one-half the cost of harvested roughage. A pound of TDN provided by concentrates cost 3.6¢, whereas the cost per pound of TDN provided by pasture was only 1.1¢.

Morrison and Ely (187) found over a 10-yr. period that the average TDN yield of bluegrass pasture was 2,226 lb. per acre. The labor involved in the production of pasture was far less than that required for the production of corn or alfalfa hay, while the TDN yield of the pasture was fairly comparable with that of corn and alfalfa hay grown in the vicinity of Lexington, Kentucky. In a comparison of low- and high-yielding cows, Vogel and Schott (292) showed that there was a loss during winter-feeding period; whereas, during the pasture period there was a profit for both high and low milk producing cows.

It is apparent that the use of well-managed pastures offers the dairyman an opportunity to increase his income.

EFFECTS OF CLIMATE ON CATTLE AND PASTURE CROPS

The summer feeding of dairy cattle involves a wide range of climatic conditions from almost ideal temperature, humidity, and rainfall to very hot and dry conditions. It is important for the dairyman to know the effects of adverse climate so that he can use improved methods for managing cattle and herbage crops under these adverse conditions.

Effect on cattle. Data have recently been reviewed concerning the effect of high environmental temperatures and humidities on the composition and yield of milk (92, 135, 246). Most of the results showed a decline in the milk yield and a change in the composition of milk when the environmental temperatures were over 80° F. but, in some cases, the adverse effects of high environmental temperatures were not pronounced (246). The effect on the composition of milk when cows are changed from barn to pasture was reviewed by McClymont (168). A depression in the fat content of milk resulted from the consumption of short, lush pastures plus fairly high levels of concentrates. The grazing of leafy oat pasture supplemented with concentrates, with limited amounts of other roughage, resulted in a depression of the butterfat percentage. These results, however, were probably not due to high environmental temperature but to high concentrate feeding.

The importance of a good ration during hot summers is indicated by Rhoad (232), who suggested that the loss of energy is, in a large way, responsible for the low production records of European cattle in the tropics. Rupel and Leighton (245) showed that cows fed a high fiber ration during hot weather produced less milk than when fed a low fiber ration. Since the two rations were balanced on a net energy basis, and not on a TDN basis, the extra heat increment produced by the high fiber ration may have been difficult to handle in hot weather. Peters *et al.* (207) reported that cows were apparently cooler on a low fiber ration because of lower body temperatures, slower respiration rates, and slower pulse rates. Holstein cows also produced milk higher in fat and solids-not-fat when fed a low-fiber ration compared to a high-fiber ration.

Using climate-controlled rooms, the Missouri workers (35, 36, 218) have found that the zone of thermal neutrality for milking cows lies between 30 and 60° F., depending on the breed, production level, and other metabolic factors. At high temperatures, humidity also becomes a very important factor, since more of the heat loss of the animal occurs through the skin and lungs. Ragsdale *et al.* (220) reported a drop in milk production with increasing environmental temperature beginning at 80° F. for Holsteins and at 85° F. for Jerseys. The decline in feed consumption with increased environmental temperature about paralleled the decline in milk production. The decline was steeper for the Holsteins than for the Jerseys.

In Puerto Rico, Basherov (25) found that the Guernsey breed is well adapted to subtropical conditions in respect to milk production, reproduction, breeding efficiency, and growth rates. Arrillaga *et al.* (15) reported that the Jersey, Brown Swiss, Guernsey, and Ayrshire breeds, in this order, are adapted to Puerto Rican climate and environment as shown by their body reactions and milk production. The Holstein breed seems to be less adaptable than the other breeds. These investigators concluded that there is nothing in the Puerto Rican environment to prevent the future development of the dairy breeds, if suitable methods of feeding, management, and protection against disease and parasites are employed. Similar results were reported by Riek and Lee (237), who exposed Jersey cows 7 hr. twice a week to temperatures ranging from 85 to 110° F.

According to Johnston (135), temperate climates usually have a 20° F. or greater difference between daily maximum and minimum temperatures. The lower night temperatures permit night grazing. Seath and Miller (255) found that cows graze about 75% of the time at night during hot weather. Payne *et al.* (205) found that cows graze 67% of the total grazing time at night. The total grazing time diminishes slightly during the hot months in the tropics. The lower night temperatures allow the cattle to recover from heat stress experienced during the day. In climate-controlled chambers, there is a tendency for milk production to decline with high environmental temperatures. When cows are allowed to adapt to hot weather gradually, as in natural climate in many hot-weather places, the reduction in milk production is delayed for about a month and then occurs only gradually (135). Johnston stated that the principal cause of "summer slump" in milk production is probably due to a decline in quantity

and quality of available herbage, probably aggravated by poor management practices. High body temperatures alone do not necessarily result in lower milk production if the animals are fed adequate TDN and their appetites remain normal. The importance of excellent forage to maintain milk production in hot weather was pointed out.

The milk production of cows kept indoors at night was less than that of cows grazed at night and day. The average herbage intakes for cows grazed day-and-night and day-only were 187 and 150 lb., respectively (291).

Cows kept in an air-conditioned chamber at 70° F. produced more milk than cows kept in an open shed (shade) when the maximum temperature was 92.7° F. (136). Roughage consumption was not affected in this study.

Shade and sprinkling. The effects of shade and sprinkling water on dairy cattle during hot weather were reviewed by Seath and Miller (254). Exposure of cows to sunshine (83–90° F. and relative humidity 61 to 80%) for 2 hr., then put in the shade, resulted in a reduction in body temperature. Sprinkling cows (body temperature 102.5° F.) reduced the body temperature by 0.54° F. after 0.5 hr. in the shade, and 1.1° F. after 1 hr. They concluded that either shade alone, or sprinkling followed by shade, was effective in reducing body temperature, respiration rate, and pulse rate. Seath and Miller (256) also showed that when cows were removed from sunshine, sprinkled with water, and then subjected to a gentle breeze, body temperatures were reduced rapidly. This may explain why rain and wind tend to cool milking cows during the summer.

Water supply. One of the problems in feeding dairy cattle in summer is the assurance of an ample supply of drinking water. This subject has been reviewed by Leitch and Thompson (159) and by Sykes (275). Cattle get considerable water when large amounts of succulent pasture forage are consumed. The total water intake, therefore, includes the water in feeds. It has been shown by several investigators that, in general, water intake follows dry matter consumption, although attempts to correlate the two have not been satisfactory.

Recent investigations at the University of Missouri include water consumption data, together with information on feed intake, environmental temperature, and relative humidity (210, 220). The effect of relative humidity on water consumption of dairy cattle was negligible at temperatures above 75° F. Winds up to 9 miles per hour did not affect water intake (35). The amount of free water consumed by Jersey cows on New Zealand pasture during the summer averaged 5.9 gal. per day, with a range of 1.4 to 11.5 gal. (50). Water intake data of milking cows at various temperatures, body weight, and butterfat percentages are shown in Table 2 (307).

Effect on pasture. The literature pertaining to the effect of fluctuations in weather upon the grasslands was reviewed by Aamodt (1), Coupland (64), Black (31), and Mitchell (182), who found a close relationship between the reduction in forage yield and drought conditions, that yield was closely related to soil moisture, and that forage yield was closely related to the ratio of precipitation to evaporation. The significance of leaf area in pasture growth was pointed out by Donald and Black (73).

TABLE 2
Water requirements of cows producing 50 lb. of FCM daily, in gallons (307)

Body weight	Temperatures (° F.)					
	40	50	60	70	80	90
(lb.)						
800	17.2	18.0	20.3	22.8	25.3	31.6
1,000	17.8	18.8	21.1	23.8	25.4	33.2
1,200	18.5	19.5	22.0	24.7	27.5	37.1
1,400	19.5	20.6	23.2	26.2	29.2	37.1
1,600	20.2	21.3	24.0	27.2	30.3	38.7

Owens (202) reviewed the papers presented at the Seventh International Grassland Congress held in New Zealand, which pertained to the effects of moisture, temperature, and light on the growth of herbage plants. It appears that plants continue to take up moisture from a soil that is getting drier, but that their ability to do so is impaired by close grazing. Such plants lose two ways; first, from loss of leaf surface for photosynthesis and, secondly, from lack of moisture. The most effective way to combat over-dry conditions on pasture herbage is to strip-graze so as to leave some pasture intact.

Investigations have shown that the mineral and protein contents of pasture plants vary widely according to species, temperature, amount of light, stage of growth, soil type, and fertilization (234, 260, 261, 272). Richardson *et al.* (234) pointed out that the protein and mineral contents of pastures from tropical and subtropical areas, in general, are lower than for pastures in temperate regions. He also stated that climatic factors affect the protein and mineral contents of a pasture; and the differences in mineral content of pastures in tropical and temperate regions may be caused by a differential effect of climate on the rate of nitrogen and mineral uptake on the one hand, and the rate of growth on the other. In tropical and subtropical climates, the growth of pasture is rapid, and frequently the plant approaches maturity within a few months after the onset of the wet season. In cool, temperate climates, growth proceeds much more slowly and the productive stage is reached only after months of slow vegetative growth. According to Harris (100), improved pastures mitigate the effects of drought and are responsible for the commencement of a soil fertility cycle by which the organic content of the soil is built up, the physical condition is improved, moisture retention is improved, and a layer of humus collects on the surface to reduce evaporation. This upward trend in the soil fertility cycle permits a wider range in the selection of plant species and results in a more rapid regeneration of pastures.

Todd (279) investigated the effects of rainy and dry seasons on the chemical composition of herbage in Kenya. Protein and phosphorus dropped to very low levels during dry periods. The studies of Reid *et al.* (228) indicate that the TDN value of herbage and, to a lesser extent, the amount consumed declines during prolonged dry periods, but increases sharply following heavy rains. Climatic conditions appear to be important determinants of quality of forage.

Irrigation. The literature dealing with the use of supplemental irrigation for the production of pasture herbage was reviewed by Trumble and Walker (284). Peterson and Hagen (208) stated that timely application of water in the correct amount is necessary to maintain summer pasture production in many parts of the world. Bateman and Keller (27, 28) reviewed the literature pertaining to irrigated pastures in arid regions and pointed out that a dairyman with a productive herd, coupled with proper management, could make a good pasture highly profitable by the use of irrigation. The establishment of more productive irrigated pastures has been a rather slow process, because it has involved the use of pasture on the better farm lands and the application of a completely different concept of pasture management—rotation grazing or strip grazing. They also pointed out the importance of using a proper pasture mixture, not only from the standpoint of nutrients but also from the standpoint of palatability. Grazing animals generally prefer younger plants to older plants, but the rate of decline in palatability with maturity varies greatly for the different species. Forage must be sufficiently palatable to be acceptable to the grazing animal, and the various components of a mixture must be utilized to such an extent that they can be maintained in proper balance throughout the life of the pasture. Species which are relatively low in palatability tend to increase with time in response to differential grazing.

Bateman and Keller (27) found that frequent applications of irrigated water are better than heavier, less frequent applications for pastures. Ladino clover, being shallow-rooted, will not persist in pasture where irrigations are not fairly frequent. During their study, pastures were irrigated five to seven times a season. In one 5-yr. period, the average number of days between applications was 26, 18, 13, 19, 17, and 18, from the first to the last, with an average of 3 to 4 in. of water per irrigation. This requires putting water over the field as fast as possible, but never when cattle are on it. The importance of maintaining the productivity of irrigated pastures through a number of seasons was stressed. In this study, pastures were grazed by cows that averaged approximately 10,900 lb. of FCM per cow per year. In another experiment, milk production for a 9-yr. period averaged 7,277 lb. of FCM per cow. Another pasture produced 8,044 lb. of FCM per acre, for a 5-yr. period. These pastures received adequate and properly spaced irrigations, applications of manure and phosphate fertilizer, and the forage was allowed to make a good recovery between grazings.

Experiments conducted by Van Horn *et al.* (289) in Tennessee gave results applicable to a large area of the United States where dry periods are frequently encountered during the summer months. The irrigated pasture produced 61% more grazing, as indicated by calculated yields of TDN, and 58% more milk than the unirrigated pasture. Distribution of grazing was greatly improved by irrigation. At the end of 4 yr., a good stand of Ladino clover remained in the irrigated pasture, but the orchardgrass was about one-fourth as thick as in the unirrigated pasture. Summer droughts are common in the region where this experiment was conducted, but the droughts which occurred while the experiment was in progress were longer and more frequent than normally would be

expected over a long period of years. Differences between irrigation and no irrigation during a series of more favorable years would probably be less than those obtained in this experiment. The authors also pointed out that farmers have other methods for maintaining or increasing milk production. Instead of investing in irrigation equipment, money might be used to provide silos for the production of additional silage for use during the periods when pastures are inadequate, or for the purchase of additional feed. The profitableness of alternative methods of increasing milk production would depend on individual farm conditions. Many farmers do not have a source of water available for irrigation.

Staten *et al.* (268) emphasized that irrigated pastures under New Mexico conditions must produce at a maximum and that rotational grazing is essential. The practice of spreading the droppings is one of the most important items in pasture management. Failure to spread droppings may result in loss of as much as one-third or more of the grazed area. Weeds and unconsumed forage should be mowed.

The results of a 10-yr. experiment on irrigated pastures at Huntley, Montana, showed that the carrying capacity of the pasture varied from 1.98 cows per acre in 1919 to 2.73 cows in 1927, with an average of 2.2 cows (148), and that an average of 6,990 lb. of milk and 266.7 lb. of butterfat were produced per acre of pasture (138.4 days of grazing). No grain was fed during the pasture period.

Tesar *et al.* (276) and Hoglund *et al.* (114) of Michigan found that the application of 3⅓ in. of water annually to an alfalfa-Ladino clover-brome-grass pasture resulted in 17.9% increase in milk production over the control pasture. The increases in milk production resulted entirely from greater carrying capacity of the irrigated pasture and were not the result of increased production per animal. Grain was fed at the rate of 1 lb. for each 4.4 lb. of milk. They concluded that in sections of the humid eastern United States, where the replacement value of hay is likely to exceed $25 per ton, irrigation might be profitable, if increases in forage production were similar to or greater than those obtained in these experiments. Irrigation of a dairy pasture may also be valuable as a means of increasing milk production per acre near metropolitan areas where land is scarce.

Munro (188) reported that the utilization of the herbage by young stock (15 to 18 mo. of age) was significantly better with irrigation. Irrigation improved the botanical composition of the pasture; the clover percentage increased on the irrigated plots, but the application of nitrogen produced some depression. This depression increased with increased moisture deficit. Milton (181) found that some species responded more than others to irrigation, and stated that timothy, ryegrass, and white clover produced relatively more growth and denser herbage under irrigation than when not irrigated. The reverse was true of cock's-foot. Munro (188) showed that nitrogen application, except for spring and fall, may be wasted on nonirrigated grass in a dry summer. The over-all increased utilization of the irrigated plots was highly significant. Munro attributed this to an increase in the palatability of the herbage and to the more

rapid breakdown of organic matter which would prevent fouling of the herbage by old dung "pats". These dung pats broke down and disappeared quite rapidly on the irrigated plots. The dung pats on the unirrigated pasture remained hard and dry throughout the summer and little breakdown was observed. Earthworms were much more active on the irrigated plots and were believed to aid in the quick breakdown of dung pats.

As pointed out by Bateman and Keller (27), one of the keys to increasing the milk production of irrigated pastures is the application of fertilizers.

Pasture fertilization. Boyd and Lessells (32) and Ward (299) reviewed the literature dealing with the fertilization of grassland. Brown and Munsell (38) showed that the protein content of timothy declined with maturity. Nitrogen fertilization increased both the percentage of protein and the yield of hay. Alfalfa out-yielded timothy, regardless of the nitrogen treatment used. In pasture-clipping studies of Ladino clover, Kentucky bluegrass, and Rhode Island bent grass, grass-legume mixtures out-yielded the grasses that had been fertilized with nitrogen in both total yield and protein. Carey *et al.* (51) reported that the fertilization of bromegrass with nitrogen resulted in a higher nitrogen percentage in the forage.

The application of nitrogen increased the yield of grasses and usually the protein content was increased. For instance, Watson *et al.* (302) stated that grassland pastures in England yielded 60% more forage from nitrogen fertilization than from no fertilizer treatment. The percentage of protein was increased by nitrogen fertilization. Also, nitrogen fertilization resulted in 13 days' earlier spring grazing and 15 days' later fall grazing than are normally obtained. Holmes (117, 118) reported that yields of grasses in England showed a year-to-year decline following continued high applications of nitrogen at rates of up to 520 lb. per acre. This decline was attributed to a potassium deficiency, brought about by the continued removal of large amounts of forage as a result of earlier application of nitrogen. Watkins (301) found that in order to obtain maximum yields from the application of urine, it must be applied to grass and not legumes. Application of phosphorus and potassium in adequate amounts is also essential for maximum herbage yield.

According to Barker (21), the productivity of grassland is largely determined by the availability of the soil nutrients within a particular environment. These can be regulated by fertilizer application, but nitrogen has the most potent influence on grass production. With good management of both land and cows, the amount of feed per acre can be increased by nitrogen fertilization at an almost linear rate up to more than 100 lb. of nitrogen per acre. Holmes and Aldrich (120) emphasized that there is plenty of evidence to indicate that cheap cattle feed can be produced by the efficient use of nitrogen on grassland. Full exploitation of this knowledge depends on a mastery of the techniques of grass utilization. Davies (68) pointed out that in New Zealand and the United States the tendency is to depend on legumes to supply a part of the nitrogen to forage crops, while in Holland and Great Britain the tendency is to rely on nitrogen fertilization. Both systems provide high yields of herbage. This problem was investigated by Wagner (293).

Levy (158) of New Zealand showed that it was possible by proper management to produce under all-year grazing 14,000 lb. of pasture dry matter containing 20 to 30% protein, and with 7,500 lb. of starch equivalent per acre per year. This is sufficient nutrients for the production of 500 lb. of butterfat per acre. A pasture producing 14,000 to 15,000 lb. of dry matter annually requires about 500 lb. of nitrogen, 1,700 lb. of potash, 7,500 lb. of superphosphate, and 300 lb. of lime per acre. He cited the work of Sears (248), which showed that a high-yielding New Zealand pedigree clover will make available from the air 400 lb. of nitrogen per acre annually, but depleted materials must be supplied.

Holmes (118) summarized the results with heavy application of nitrogen upon pasture yields at the Hannah Dairy Institute in Scotland under good management conditions. The addition of nitrogen in 100-lb. increments up to 350 lb. per acre showed a straight-line increase in dry matter yield, and only a slight decrease in efficiency of dry matter production up to 500 lb. per acre. Holmes concluded that increased yields per acre of dry matter, Starch Equivalent and protein are almost linearly related to nitrogen supply up to about 300 lb. per acre. Clover pastures can contribute the equivalent of 150 to 200 lb. of nitrogen per acre. Each cow contributed 0.5 lb. of nitrogen daily in the form of urine and feces, or nearly half of the required nitrogen. Holmes stressed that if these results are to be obtained under practical conditions the efficiency of utilization must be high. Rotational pastures are essential, strip grazing is desirable, and good conservation methods are required. Holmes and Aldrich (120) compared the cost of Starch Equivalent produced from the use of nitrogen fertilizer on grass with the cost of other feeds and concluded that cheap cattle feed can be produced by the efficient use of nitrogen on grass.

Ramage *et al.* (221) of New Jersey applied nitrogen in 50-lb. increments up to 450 lb. per acre. The dry matter yield at 50 lb. per acre was two tons containing 12% protein. These yields gradually increased with each 50-lb. increment up to 4.5 tons, containing 20% protein at the rate of 450 lb. of nitrogen per acre. Nitrogen application of 100 lb. per acre gave the greatest yield of dry matter and protein per pound of applied nitrogen.

Sprague and Garber (267) studied the effects of various harvest treatments and nitrogen fertilization on Ladino clover grown in combination with orchardgrass and smooth bromegrass. The application of 60 lb. of nitrogen per acre increased grass yields and reduced clover growth. The fertilization of pastures with nitrogen, phosphorus, and potassium increased forage yield (147). Crude protein, phosphorus, and potassium percentages in the forages were increased. Mid-season application of nitrogen increased the nutritive value of the forage. Soil types had greater effect on the chemical composition of the forage than did the application of lime, nitrogen, and phosphorus as fertilizers (286). Phosphorus and potassium fertilizers tended to increase clover population. Forage yields from grasses were greatly increased by nitrogen fertilization (274). The importance of using fertilizers on pastures was pointed out by Abell (3).

According to Holt *et al.* (123), the application of fertilizers to both summer annual and perennial grasses resulted in increased yields. Nitrogen, phosphorus,

and potassium were found to be necessary for good pastures in Texas. The effects of nitrogen fertilization on composition and dry matter production of grassland were also investigated by Ferguson and Terry (82).

In a long-continued hay-pasture experiment in Connecticut, lime, phosphorus, and potassium were necessary for the maintenance of high proportions of volunteer white and red clovers (37). The quality of forage from the nitrogen-treated plots was inferior to that from the more clover, no-nitrogen area, according to the preference of yearling heifers. Attention was called to the importance of legumes in grassland farming which has been emphasized by many investigators. There is no doubt that legumes save tremendous amounts of fertilizer nitrogen. The dry matter and protein yields of Kentucky bluegrass and Rhode Island bentgrass, lawnmown when 4 in. high, were larger when seeded with Ladino clover than when seeded alone and given 84 lb. of nitrogen per acre.

According to Lassiter *et al.* (155), the application of manure each year on bluegrass pasture increased the yield of TDN per acre from 13.0 to 22.6% over that of the control pasture. Various manure treatments had little or no effect on the crude protein, but tended to lower the dry matter content of the forage. Van Horn and Dawson (290) stated that the application of manure to permanent pastures increased TDN production 38 to 41%. Eby *et al.* (77) and Nevens (193, 195) also observed that the use of barn manure increased the yield of pastures.

Salter and Yoder (247) concluded that it was more profitable to produce both protein and dry matter by the application of fertilizers to rotating pastures than it was to buy these two materials. Archibald and Nelson (10) also found that intensive fertilization and proper grazing not only increased the yield of dry matter but also increased the feeding value. According to Seath (250), the annual returns per acre over and above the cost of grain fed and fertilizer used over a 4-yr. period averaged $36.73 for unfertilized pastures and $42.84 for fertilized pastures. The best results were secured when the following general procedure was followed in improving a pasture: (a) arrange terraces in pasture to control erosion; (b) apply lime to correct acidity; (c) thoroughly prepare seedbed through plowing, disking, harrowing, and rolling or packing; (d) apply fertilizer, especially phosphorus; (e) seed with adapted clovers and grasses, (f) divide pastures so that grazing can be controlled by rotating cattle from pasture to pasture; (g) control weeds by mowing pastures from two to four times a year; and (h) have water and shade available in all pastures.

PASTURE EVALUATION

Most data available on the amount of milk produced from pasture herbage are given for the entire pasture season. This review, however, is primarily concerned with milk output from summer-grown herbage. Raymond (222) stated that the total annual yield from a pasture is relatively less important than the distribution of the yield between different months of the year. Total annual yield is always dominated by May-June production but, in terms of practical grazing management, herbage production during the other months is of greater

importance. Persistence, palatability, and succulence are more important than total yield. In determining the feeding value of a pasture, the potential level of the grazing animals and not the pasture may be the limiting factor (130). Raymond (222) is of the opinion that new techniques for measuring and comparing pasture outputs should be used over relatively short periods, rather than over the whole season, as has generally been done. Such methods, however, are subject to large errors and are very sensitive to skill in pasture management.

Milk yield. The difficulties in using milk yield as a yardstick in measuring grassland output have been pointed out by Castle (52, 53, 54) and Cox (65). The difficulties include variations in stocking rate, potential milk production of cows, variations in the herbage due to growth and changing weather conditions, and variation in losses due to trampling.

One of the requirements stipulated as basic to high production is efficient harvesting of the pasture herbage (176). Experimental results suggest that the stocking rate is of major importance in this connection. In experiments by Hancock (93), the other factors were kept constant and the stocking rate varied. Thirty cows were used in each of two groups. The low rate stocking group required 30 acres and produced 320 lb. of butterfat per acre, while the high rate group required 18 acres and produced 453 lb. of butterfat. Wallace (296) obtained similar results with two groups of cows. The low rate group required 60 acres of pasture and produced 213 lb. of butterfat per acre, whereas the high rate group required 32 acres and produced 269 lb. of butterfat.

According to McMeekan (179), a stocking rate as high as one milking cow per acre has resulted in an estimated minimum invisible wastage amounting to 35% of all the herbage actually available. Over-stocking may decrease the yield because of close grazing and frequent defoliation, and have long-term adverse effects on the sward.

A review of the early literature dealing with treading (trampling) was made by Edmund (78). Most investigators reported losses of pasture forage due to treading, which altered plant composition and brought about physical and chemical changes in the soil. Increases in treading produced significant and progressive reduction in the yield of all species. Treading loss, when compared with that of mowed crops, amounted to 10 to 15%. Riddet *et al.* (236) of New Zealand attempted to increase milk production from pasture to a level approaching 500 lb. of butterfat per acre. After 5 yr., which included three abnormally dry summers, production had reached only 350 lb. of butterfat per acre. Treading damage may have been the major limiting factor. They suggest that the on-and-off system of grazing might be explored in order to reduce treading. It has been shown that dairy cows can satisfy their appetites in relatively short grazing periods. Then, too, there is a possibility of selecting cows that bring about reduced treading losses.

In a comparison of the nutritive output of a pasture, that the potential level of the grazing animals and not the pasture may have been the limiting factor was suggested by Ivins *et al.* (130). Cox *et al.* (65) used a single cow on each plot, to relate individual pasture intake and milk production. A significant

linear relationship between milk yield and estimated dry matter intake over a 3-wk. period was found.

Composition and digestibility. In the past, most of the methods of estimating pasture herbage intake and its composition and digestibility, and dry matter intake of pasture herbage and estimated nutritive value, were based on the clipping method. The chemical composition, digestibility, and yield of forage crops as feeds for dairy cattle depend on the climate, soil fertility, amount of water available to the plants, botanical composition, and state of maturity of the plants. Some of the early work was reviewed by Huffman (127). Sullivan and Garber (272) reviewed the chemical composition of pasture plants with some reference to dietary needs of grazing animals.

The historical results of Woodman and associates (1926-1938), as compiled by Huffman (127), are shown in Table 3. The effects are shown of increased maturity of both grass and alfalfa on the amount and digestibility of protein and crude fiber. Pasture grass cut every 3 wk. contained 21.1% of protein (dry basis) with a coefficient of digestibility of 79. The feeding value of this pasture was equal to pasture cut at weekly intervals. For all practical purposes, pasture grass cut every 5 wk. was almost as nutritious as the crop cut earlier, and was far superior in productive energy to good meadow hay. Alfalfa cut in prebud contained 25.3% protein and had a coefficient of digestibility of 84. The crude fiber content increased from 22.1% in the prebud stage to 29.7% in the flower stage. The energy value declined from a S.E. (Starch Equivalent) value of 59.8 in the prebud to 42.4 in the flower stage.

Phillips *et al.* (210) studied the changes in chemical composition of timothy tops and found that the percentages of protein decreased, whereas methoxyl, pentosans, crude fiber, lignin, and pectic substances increased with maturity. Waite and Sastry (295) reported that timothy decreased in percentage of protein and increased in fiber with maturity. Armstrong *et al.* (13) observed that crude fiber, lignin, and crude cellulose increased to the seed stage in four different grasses.

According to Laughlin (157), bromegrass harvested at different times throughout the summer in the Matanuska Valley of Alaska showed a decline in protein percentage from 27.4 on May 28 to 4.2 on September 25. Yields of protein were highest in early July, at the bloom stage. Nitrogen application increased the protein content and herbage yields. Heavy nitrogen treatments were more effective when accompanied by phosphorus and potash fertilizers.

Using indicator methods for estimating the amount and composition of grazed forage, Reid *et al.* (228) found that first growth contained 31.0 and 18.8% of crude protein and crude fiber, respectively, on the dry basis, whereas full bloom contained 26.1 and 34.8% protein and fiber, respectively. The grazed herbage contained more protein and less fiber than comparable clipped herbage. Using the same method, Richards and Reid (233) determined the chemical composition of pasture forage, both hand-fed and grazed, in the vegetative, boot-to-early-head, and full-bloom stages of growth. Although the crude protein content and digestibility of both hand-fed and grazed forage decreased with maturity,

TABLE 3

Composition and feeding value of pasture grass and alfalfa cut at different stages of maturity on the dry basis (127)

	Protein	Coefficients of digestibility of protein	Digestible protein	Fiber	Coefficients of digestible fiber	N.F.E.	Coefficients of digestible N.F.E.	Coefficients of digestible organic matter	Starch value
	(%)								
Pasture									
Cut weekly	24.7	81	20.0	15.4	76.7	44.8	81.5	79	67.7
Cut every 2 wk.	23.5	80	18.8	15.9	81.3	44.5	82.8	80	69.8
Cut every 3 wk.	21.1	79	16.7	17.2	79.2	46.7	83.5	80	69.4
Cut every 4 wk.	19.4	76	14.7	21.2	81.7	46.3	82.8	80	67.1
Cut every 5 wk.	18.3	74	13.6	21.9	82.1	47.2	79.1	77	64.7
Good meadow hay	13.8	63	8.7	25.8				65	45.2
Poor meadow hay	7.5	45	3.2	33.5				52	24.0
Alfalfa									
Prebud	25.3	84	21.3	22.1	63.7	38.1	79.8	76	59.8
In bud	20.4	76	15.5	23.9	46.3	42.2	74.2	66	50.8
In flower	17.2	74	13.0	29.7	42.6	39.4	70.2	60	42.4
Average alfalfa	14.2	74	10.5	29.5	50.8	40.9	73.7	....	33.0

the grazed forage contained more protein of greater digestibility. Crude fiber, and cellulose content and digestibilities, decreased with advanced maturity, but the grazed forage was lower in both constituents and higher in digestibilities than the hand-fed herbages. The digestibilities of the NFE and hemicellulose contents of the grazed forage were higher than the hand-fed forage at the various stages of growth. Waite *et al.* (294) measured the output of strip grazing under conditions that largely eliminated the errors suggested by Linehan (160).

The plucking of forage from protected areas until it is similar in appearance to grazed areas was suggested by Sears (248), but allowances for herbage growth during the grazing period are difficult and unsatisfactory.

As has been pointed out, the production of pasture in terms of growth and/or milk production is the best measurement of pasture output. Methods used in pasture studies have been reviewed and recommendations made by a committee on pasture research (204). These include the use of "unit grazing days," TDN output, moving techniques, etc.

Linehan *et al.* (160) showed that over a period of 3 yr. a clipping method gave an output of 101% of that obtained from animal data. During short grazing periods, however, the variation of the measurement of pasture output by mowing techniques and animal output data has not been satisfactory, probably because of selective grazing. Consequently, there is increased interest in methods of determining the amount, chemical composition, and digestibility of grazed herbage.

Indirect methods. Digestibility-indicator methods have shown the most promise. These involve the determination of the indigestibility of the pasture and the fecal output. The indicator for obtaining the indigestibility of grazed herbage should be a naturally occurring substance in the pasture forage, whereas an indigestibile external indicator should be used to measure the fecal output. The combination of the two values should give the total intake.

Two methods are used to measure the indigestibility of herbage: (a) the ratio technique, which requires a naturally occurring indigestible indicator in the forage and the determination of the indicator in both herbage and feces, and (b) the fecal-index technique, in which the indicator is not necessarily indigestible and is measured only in the feces.

Commonly used internal indicators are lignin, methoxyl groups, crude fiber, nitrogen, and plant chromogens. The principal external indicator for measuring fecal output is chromic oxide (Cr_2O_3). Several reviews describing indicator methods are available (140, 223, 227).

Most investigators have found that lignin ratios are unreliable indicators of digestibility. On the other hand, Ellis *et al.* (79) reported that lignin was not digested in any significant amount. Kane *et al.* (141) found complete recovery of lignin and chromic oxide and concluded that they are suitable for digestive studies. Ely *et al.* (80) used the method of Ellis *et al.* (79) and concluded that lignin recoveries were lower than are desirable for an inert tracer material. According to Balch *et al.* (20), crude lignin as determined by the method of Armitage *et al.* (11) showed negligible digestibility and that it should be satis-

factory as the basis of a ratio technique for the dairy cow. It would appear that lignin could be employed as a fecal indicator when improved methods of lignin determination are devised.

Several research workers have used fecal nitrogen or protein in the estimation of forage digestibility. Gallup and Briggs (86) observed that the excretion of nitrogen by steers consuming hay was related to dry matter intake. Raymond (224) and Lancaster (151) also reported that the concentration of nitrogen in the feces of animals was related to the amount of grass consumed. Forbes (83) and Homb and Breirem (124) showed that the amount of nitrogen excreted per unit of dry matter intake was not constant, but was related to the nitrogen content of the forage. As the result of more recent studies, Lancaster (151) modified the method by relating the ratio of feed consumed to the nitrogen content of the feces. His formula may be used to determine both digestibility and forage intake. Raymond *et al.* (224) found that the percentage of organic matter digested by sheep was related to the percentage of nitrogen in the ash-free dry matter of the feces. Milford (180) showed a highly significant positive correlation between dry matter intake and total nitrogen in the feces. Digestibilities of organic matter calculated from fecal nitrogen showed fairly good agreement with values determined by conventional methods for unfertilized grass, but were high for nitrogen-fertilized grass.

A system of utilizing naturally occurring plant chromogen(s) as an internal indicator in pasture evaluation has been worked out by Reid *et al.* (231), who found that digestion coefficients obtained by the chromogen-ratio compared favorably with results obtained from conventional digestion trials. In later studies, Reid *et al.* (230) found that selective grazing and hand-plucked forage samples were not identical with the herbage grazed by the animal and that 100% recovery of ingested chromogen was not obtained when grain was fed in digestion trials with cows. It was noted, however, that there was a relationship between fecal chromogen concentration and forage chromogen concentration (and, hence, digestibility). For successful application, this method did not require sampling the grazed herbage or complete recovery of ingested chromogen.

In further studies, Smith and Reid (263) improved the chromogen technique. Lancaster and Bartrum (152) and Kane and Jacobson (139) found that extracts exposed to strong light or to moderate amounts of light for as long as 6 hr. did not increase the pigment in the fecal extracts. Satisfactory results were obtained when this method was compared with conventional digestion trials (139, 156, 231, 263).

Ingested chromic oxide (Cr_2O_3) was completely recovered from the totally collected feces of several species of animals, and when used in the ratio technique obviated the need for data on the amount of feed consumed and feces voided (227). If Cr_2O_3 passes into the feces in a predictable manner, the total collection of feces could be supplemented by periodic grab samples. Hardison and Reid (98) found that the Cr_2O_3 excretion-time patterns were remarkably similar for steers and cows grazing different pastures during different seasons. Smith and Reid (263) estimated the output of feces equally well when the Cr_2O_3

was administered in capsules or in concentrate feeds. The effectiveness of the procedure for measuring fecal output was the same, regardless of whether Cr_2O_3 was given at 6 A.M. or 4 P.M., or at both times.

Davis *et al.* (69) observed a day-to-day fluctuation in Cr_2O_3 excretion in cattle which was overcome by ten-day collection periods. Putnam *et al.* (217) concluded that the time of Cr_2O_3 administration was of primary importance in respect to the time-concentration relationship of fecal Cr_2O_3, and that so-called physiological, diurnal effects were of little importance. A 12-hr. sampling procedure would be more accurate than unequal fecal sampling times.

To circumvent the use of total fecal collections with grazing animals, Hardison and Reid (98) investigated the possibility of using small, randomly procured fecal grab samples in a limited trial, and found close agreement between the chromogen content of samples taken in the morning and afternoon with those taken from the totally collected feces. Other workers reported large daily variations in chromogen excretion and without a definite pattern. However, fecal grab samples have given results comparable to those obtained by the total collections in several trials, according to Anthony *et al.* (9) and Kane *et al.* (138). From data concerning dry matter intake obtained by the chromic oxide–chromogen technique (98, 231), it can be assumed that grab samples are satisfactory in the estimation of forage digestibility.

APPETITE FOR PASTURE HERBAGE

The economic production of milk is based on the use of high-producing cows, that will consume large amounts of high-quality and high-yielding roughage. The feed capacity or appetite of an animal is the total amount of dry matter consumed when offered as much as it cares to eat (190). Many factors affect the appetite of cattle, such as size, health, inheritance, psychological factors, exercise, and milk production; also, factors inherent in the feed and environment, such as palatability, amount of feed offered, bulk of the ration, balance of the ration, and environment temperatures. Palatability is frequently used to denote appetite. Actually, palatability is tested by the use of the cafeteria method, whereas appetite is measured in the weight of dry matter consumed. There are several reviews on this subject (94, 127, 171, 280). Palatability as an index of nutritive value of roughage has been overemphasized, since it is not always a criterion of dry matter consumption.

Shaw and Atkeson (257) determined the comparative palatability of four cereal pastures with dairy cows. The cows grazed 52% of their time on Balbo rye, 24% on common rye, 18% on winter wheat, and 6% on winter barley. The palatability of a pasture composed of several species of grasses is important. Under such conditions, some species become less palatable than others.

Hancock of New Zealand (94) reviewed the early work on animal behavior studies that apply to dairy cattle. Hancock (95) used lactating identical twin cows and found that an outstanding feature of the grazing behavior was its variability, due to external and internal conditions. Of the external factors, climate

in a temperate zone was relatively unimportant. The quantity and quality of the herbage offered were important in modifying the grazing behavior of dairy cattle. Under adverse pasture conditions there was, in general, an increase of total working time (grazing + ruminating). Grazing time increased with the scarcity and quality of forage, while ruminating time was prolonged by poor-quality grass. The adaptation of grazing habits to new conditions was very rapid. The feed requirement for milk production was the most important internal factor determining grazing time. Hardison *et al.* (96) have also reviewed the recent studies on behavior of dairy cattle. The problem of determining accurately the feed consumed by grazing animals was reviewed by Aamodt *et al.* (2). One of the first reliable studies on pasture grass consumption was that of Helms (105), who reported that two lots of six cows each were tethered on grass within large, measured circles. At the end of the day, the grass left within the circle was cut and weighed. The trial lasted 14 days. The cows consumed about 155 lb. of grass per head daily. Graves *et al.* (89) fed grass clipped from irrigated pastures to four Holstein-Friesian cows for periods of 15 to 55 days. The average consumption of individual cows for the entire period varied from 122 lb. for the smallest cow to 152 lb. for the largest. The results obtained with one cow which was switched back and forth from pasture to grass clippings indicated that cows do not consume as much dry matter while grazing as when fed grass clippings. Woodward (310) concluded that a cow will graze about 6% of the immature grass on one acre of pasture, or 30 to 35 lb. of dry matter daily, depending on its size. Husted-Anderson (129) calculated the dry matter intake of pasture from collected feces. The cows weighed from 1,100 to 1,320 lb., produced from 17.6 to 41.8 lb. of milk daily, and consumed 24.2 to 39.6 lb. of dry matter per cow daily from pasture. The feeding of concentrates caused a significant decrease in dry matter intake from pasture. Night grazing increased pasture dry matter intake 20% over day grazing.

MacLusky (161) found that cows consumed 2.0 to 2.2% of their live weight of pasture dry matter, with an average of about 2.1%. According to Hoskins and Line (125), the daily herbage dry matter intakes of 20.8 to 30.3 per cow, equivalent to 20.8 to 25.4 lb. per 1,000 lb. live weight, were obtained for the daily and four-day folding methods.

According to Cox *et al.* (65), experimentation with grazing, lactating cows involved difficulties associated with the usual variations in milk production, variability of the pasture due to growth, and changing weather conditions. They pointed out that a limiting factor would often be the availability of cows giving sufficient milk at suitable stages of lactation. In this investigation, the possibility of pasture evaluation in terms of milk production was examined, using individually grazed cows on a ryegrass-clover pasture. There was a significant linear relationship between mean milk yield and estimated dry-matter intake over a 3-wk. period. The average mean fresh forage intake for 16 cows was 188 lb. per day. The highest value for a single cow was 233, and the lowest was 125 lb. per day. The mean dry matter intake was 28.2 lb. (22.4 to 32.8 lb.) and the mean FCM yield was 32.0 lb. (19 to 48 lb.) per day.

Using the clipping method, Phillips and Baker (209) reported that cows ate 86 lb. of Ladino clover–orchard grass mixture; 70 lb. of white clover–bluegrass mixture, and 72 lb. of lespedeza-orchardgrass mixture per cow daily. The large amount of pasture dry matter consumed was 45.1 lb. daily, reported by Hill and Lundquist (109). According to Kley (146), the highest dry matter intake was obtained on grass 8 to 9 in. high and yielding about 2,500 lb. per acre. Duckworth and Shirlow (76) concluded that dry intake on pasture depended on the crude fiber content; as the percentage of dry matter increased in the pasture plants, the dry matter intake decreased.

There was a highly significant relation between body weight of growing cattle and dry matter intake, according to Martin *et al.* (166). Dry matter intake increased by 3 lb. daily for each body weight increase of 100 lb., provided sufficient herbage was available to satisfy the appetite.

The effect of the level of milk production and calculated TDN consumed from pasture was discussed by McCullough (171). He also called attention to the relation of pasture dry matter intake to dry matter yield per acre. He cited data from the Netherlands to show that cows consumed 44.0 lb. of dry matter when pasture yielded 2,500 lb. of dry matter, and only 33.0 lb. when the yield was 1,568 lb. per acre. With a yield of 572 lb. of dry matter per acre, the intake was only 15.4 lb. per cow per day.

According to Castle (53), the average daily consumption of ryegrass and cock's-foot herbage dry matter per cow and per 100 lb. of live weight was 32.9 and 2.9 lb. in 1940, 30.7 and 2.9 lb. in 1950, and 26.6 and 2.5 lb. in 1951. Using the chromogen technique, Noller *et al.* (197) reported that three Holstein cows produced 54.9, 40.0, and 37.9 lb. of FCM daily for 84 days and consumed an average of 225 lb. of grass daily, with coefficients of digestibility of 75, 72, and 70%, respectively. In England, it is assumed that cows on high-quality pasture consumed 30 lb. of dry matter daily per 1,000 lb. of live weight (309).

Selective grazing by dairy cattle. In the past, most of the data showing the chemical analysis and digestibility of pasture forage have been based on clipped samples, although it was assumed that animals graze selectively. Attempts have been made to simulate grazing by plucking selected pasture forage to determine the chemical analysis and feeding value (143). The technique developed by Reid and associates (34, 99, 230), for estimating herbage intake and digestibility by grazing animals, has made possible the study of the effect and degree of selective grazing. The earlier work was reviewed by Hardison *et al.* (99). Some of the results of an experiment with growing steers are shown in Table 5. These data show a marked degree of discriminate grazing of the herbages. The forage selected by the grazing animals was higher in crude protein, ether extract, and mineral matter, and lower in crude fiber, than the whole clipped herbage available for consumption. All the constituents of grazed herbage were more digestible than those of the clipped herbage fed to confined steers. The digestibility of dry matter and the amounts of crude protein and ether extract decreased, and the amount of crude fiber increased, as first-growth forage became more mature. Although aftermath herbage contained a high level of crude

TABLE 4

Chemical composition and digestibility of constituents of forage hand-fed and grazed (233)

Constituent	Vegetative stage				Boot-to-early-head stage				Full-bloom stage			
	Hand-fed		Grazed		Hand-fed		Grazed		Hand-fed		Grazed	
	Chemical composition	Digestibility	Chemical composition	Digestibility	Chemical composition	Digestibility	Chemical composition	Digestibility	Chemical composition	Digestibility	Chemical composition	Digestibility
	(%)											
Dry matter	.....	72.9	.....	76.9	.....	66.3	.....	69.4	.....	58.0	.....	58.2
Crude protein	16.1	73.7	18.0	77.8	12.0	66.0	13.6	69.5	10.4	60.8	11.5	61.1
Ether extract	4.8	53.7	5.7	60.8	4.3	56.1	5.1	60.4	3.3	30.3	3.8	30.7
Crude fiber	25.2	73.6	22.8	77.5	27.3	65.0	25.4	68.0	31.8	59.1	29.4	59.4
Nitrogen-free extract	46.4	76.5	46.2	80.3	49.4	69.0	47.8	72.1	49.1	59.9	49.2	60.4
Ash	7.5	58.9	7.3	64.6	6.9	50.5	8.0	63.1	5.4	47.3	6.1	46.0
Lignin	5.8	−11.7	5.2	5.7	6.7	−4.9	6.9	5.3	8.6	−9.0	8.5	8.0
Cellulose	29.9	77.8	30.0	81.3	32.0	68.6	30.4	71.6	33.4	61.0	29.8	61.5
Hemicellulose	17.2	76.5	18.2	80.1	19.2	65.8	17.8	69.0	20.2	61.3	23.7	61.7
Starch	8.2	96.0	8.6	96.0	7.8	96.0	6.9	96.0	7.7	96.0	7.7	96.0
Sugars	5.5	98.9	4.9	99.0	6.8	99.0	4.2	99.1	5.2	99.2	12.4	99.2
Pectins	2.2	87.8	1.6	92.3	1.8	75.1	2.0	77.7	1.1	68.3	1.1	68.7

TABLE 5

Composition and digestibility of herbage selected by grazing steers compared with that of whole herbage cut from the same source (99)

Trial No.	Steer No.	Composition of herbage dry matter					Digestibility				
		Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	Dry matter	Crude protein	Ether extract	Crude fiber	N.F.E.
		(%)									
1949 Forage 1											
1	0	19.5	6.4	21.6	45.5	7.0	78	79	62	78	81
	36	17.4	5.2	23.0	46.7	7.7	77	77	60	77	80
	39	17.0	5.4	23.9	46.6	7.1	76	77	60	77	80
Whole herbage		16.1	4.8	25.2	46.4	7.5	73	74	54	73	77
1950 Forage 1											
1	4	22.9	5.0	26.5	39.1	6.5	75	79	47	76	76
	5	25.0	6.2	25.0	36.9	6.9	75	80	48	77	77
	6	24.7	6.8	27.7	33.4	7.4	76	81	50	78	79
Whole herbage		20.2	4.7	27.9	39.4	7.8	73	78	42	74	74
1950 Forage 2											
1	10	26.0	3.9	24.3	40.8	5.0	72	80	37	70	76
	11	23.4	3.4	25.5	43.4	4.3	70	78	31	67	75
	12	24.7	4.4	22.9	43.0	5.0	75	82	42	72	79
Whole herbage		22.0	3.8	26.9	39.9	7.6	72	80	37	70	77
1951 Forage 2											
1	29	26.0	7.0	18.2	34.8	13.9	68	78	62	56	69
	30	23.1	6.2	23.4	34.2	12.6	65	78	61	52	68
Whole herbage		17.0	3.1	34.9	38.5	8.7	57	73	54	45	63

protein and a low level of crude fiber, it was somewhat less digestible than the first growth of forages of comparable composition. These workers concluded that this investigation demonstrated that the chemical composition of clipped herbage was an unreliable index of the chemical composition of herbage selected by grazing animals, and that the digestibility of clipped herbage was an uncertain value and could be quite misleading in regard to estimating the value of pasture under grazing conditions. On the other hand, Murdock *et al.* (189) used three Holstein heifers and presented data which indicated no apparent decline in digestibility from early spring to aftermath forage.

REQUIREMENTS OF COWS ON PASTURE

The principles that apply to the rationing of dairy cows during the winter can not be applied with the same degree of accuracy to cows grazing summer herbage. Energy is the most important dietary constituent in the dairy cow's ration, because of the large amount needed for maintenance, milk production, and grazing. This subject has recently been reviewed (226, 297). Reid *et al.* (228) used the Cornell and New Zealand data and worked out a formula for calculating the TDN requirement for maintenance for cows on pasture. A 1,000-lb. cow requires 7.9 lb. of TDN for barn maintenance, according to Morrison (186). On pasture, the maintenance requirement is about 40% more, or 3.4 lb. of TDN for a total daily requirement of 11.3 lb. of TDN.

According to Barker *et al.* (22), the energy expended in grazing is affected by a number of variables such as the availability of the grass, grazing management (continuous, rotational, strip), climatic conditions, and the incidence of insects, parasites, and other disturbing factors which would increase the energy requirements or reduce the efficiency of feed conversion. As the energy expended is unavoidable and can not wholly be attributed to the nature of the grass, it is recommended that this item be disregarded, since a uniform allowance would be inappropriate. A 1,000-lb. cow requires 6.5 lb. of S.E. for maintenance and a 2.5 lb. S.E. for each 10 lb. of milk testing 4% fat.

Castle (53) reported that the S.E. required for grazing was either 1, 2, or 3 lb. per cow per day, as judged by the appearance of the animal. When strip grazing, 1 lb. of S.E. was allowed per cow daily. According to Woodman (309), the scientific evidence of the magnitude of the energy expended in grazing is scanty, but it would seem to vary from 1 lb. of S.E. per day on pasture of good quality and dense growth to as much as 3 lb. per day on poor pastures carrying sparse growth herbage.

Good pasture herbage usually contains more digestible protein than is required for liberal milk production. Hardison *et al.* (99) showed that cattle select the parts of plants higher in crude protein which are more digestible than clipped forage. According to Pratt and Davis (212), a grain mixture containing approximately 10% protein is adequate to support an average milk production of 30 lb. of FCM on good pasture. When pasture herbage is mature or dried up, it is advisable to use barn-feeding requirements.

The mineral requirements for growth and milk production used for winter-feeding probably apply to summer-feeding. The requirements and brief discussions of known mineral requirements are presented by the National Research Council (192). In certain areas of the world, cattle may need either phosphorus, cobalt, copper, or iodine as a supplement. Sometimes more than one deficiency may be manifested by cows on pasture. Grass tetany associated with disturbed magnesium metabolism is a spring disease and does not usually occur among summer-grazed cattle.

When pastures are not available, winter-feeding information should be used.

PASTURE SUPPLEMENTS

The best supplement to permanent pasture in areas subject to drought is a good temporary pasture especially adapted to local conditions. In many areas, Sudan grass meets this need. The value of supplemental pastures has been pointed out by many investigators, including O'Neil (200).

The amount of milk produced per day by cows on pasture as the only feed appears variable. The results of McCullough (170) indicate that dairy cows could be maintained in excess of 40 lb. of milk per cow daily on pasture alone, provided the dry matter is at least 70% digestible. According to Castle (55), good leafy pasture herbage can supply all the nutrients necessary for cows yielding 60 lb. of milk a day. Turner (285) gave the same estimation. Experiments by Hill and Lundquist (109) showed that cows produce up to 50 lb. of milk per day on pasture alone. As has been pointed out, high-quality pasture herbage is capable of supplying the needs of good producing cows for the production of about 50 lb. of milk (2 lb. fat). Further experiments are needed to determine the effect on milk production of adding various supplements to such pastures.

In many areas, the mid-summer slump in milk production is a common seasonal occurrence. Efforts to prevent or minimize the reduction in milk production include the use of hay, silage, or concentrates as pasture supplements. Seath *et al.* (252, 253) fed hay to prevent summer slump in milk production. In a preliminary trial, insignificant increases in milk production were secured when 9 lb. of white Dutch clover hay was fed daily. In a second trial, milk production was significantly increased when hay was full-fed on pasture, but it was unprofitable. In Kentucky (7), there was no appreciable increase in milk production or body weight when alfalfa hay was fed free choice to Holstein and Jersey cows on pasture during mid-summer. Seath and Elliott (251) found that feeding either corn silage or an extra amount of a grain mixture to Holstein and Jersey cows aided in preventing the late summer decline (August 19-September 11) in milk production. Since grazing conditions were better-than-average, the benefits were not large enough to be profitable. Seath (249, 251) reported some improvement in the level of milk production when alfalfa silage was fed as a supplement to cows grazing a mixture of Kentucky 31 fescue and Ladino clover. Additional grain fed as a supplement to Kentucky 31 fescue pasture increased milk production, probably because of lack of palatability of the fescue.

According to Seath *et al.* (252, 253), a group of cows on bluegrass–white clover pasture was fed all the alfalfa hay they would eat for 2 hr. twice daily, and another group was on the same pasture without hay. Both groups received grain. The dry matter intake from pasture was estimated by combining the chromogen(s) and chromic oxide techniques. The cows receiving hay consumed 29.6 lb., and those without hay 26.6 lb., per cow daily of total dry matter. The hay supplementation did not increase milk production, but increased the gain in body weight 50 lb. per cow during the experiment.

Cole *et al.* (58) investigated the influence of supplementing bluegrass–white clover pasture during the summer with three groups of cows. Group 1 was a control, Group 2 was supplemented with alfalfa hay, and Group 3 received alfalfa silage as a supplement to pasture. Neither supplement increased dry matter intake; however, the portion of the dry matter received from pasture was reduced 33.9% as a result of hay feeding and 13.1% as a result of silage feeding. The three supplement groups consumed 27.4, 18.1, and 23.8 lb. per cow daily, respectively. The group fed alfalfa silage consumed 4.7 lb. less dry matter as silage than the hay cows. The supplementation with silage or alfalfa hay did not affect milk production or body weights. Monroe (183) reported that hay fed to a group of cows on improved bluegrass pasture increased milk production (10 lb. per cow daily), compared with a group that did not receive hay on pasture.

Nordfeldt and Hansson (198) investigated the effect of adding supplements to dairy cows on pasture with the following results: hay (0.9 feed unit per cow) daily) as a supplement to pasture over a 6-yr. period increased FCM 1.5 lb. daily, compared to a control group with no supplement. At the current prices for feed and milk, the increase in milk production was not economically advantageous. These investigators do not recommend the feeding of supplementary feeds to cows on pasture—up to those producing 33 to 44 lb. of milk daily.

Hancock (93) used identical twins to study three levels of pasture nutrition with three groups of cows. Group 1, one milking cow per acre plus 1 lb. of concentrate for each 5 lb. of milk produced; Group 2, one cow per acre with no concentrates; and Group 3, one cow per 0.6 acre with no concentrates. The groups were rotationally grazed on fresh pasture on a 14-day rotation. The Group 1 cows produced 45% and the Group 2 cows 16% more FCM than the Group 3 cows. Per unit area, however, the Group 3 cows produced 30% more milk than the Group 2 cows. The greater milk yield of the Group 1 cows over the Group 2 cows was attributed mainly to their capacity to maintain a high grass intake along with concentrates, so that the total dry matter intake was maintained at a high level. The Group 2 cows consumed 10% more organic matter than the Group 3 cows, and the Group 1 cows consumed 5% more than the Group 3 cows. The intake of organic matter from grass for the three groups was in the same proportions as the ruminating time, which is a good index of the relative grass intake when cattle are grazed on pasture of similar quality. The results indicate clearly that pasture herbage is not a perfect diet for dairy cattle, since concentrates are capable of giving a substantial increase in milk yields when added to a diet of high-quality pasture. It seems likely that this effect is due mainly to the

fact that dairy cows are capable of consuming some concentrates even when they have satisfied their appetite for pasture herbage, and not to specific constituents or associate effects of the concentrates. If this is true, it is obvious that one of the limiting factors to maximum milk production from good-quality grasslands is the volume of grass which cows can consume. The reasons and remedies for this seem worthy of further investigation. The results also indicate that pasturing dairy cows at a stocking rate as high as one cow per acre may result in an invisible loss of at least one-third of all herbage grown. A higher stocking rate is an obvious way of reducing this wastage.

In the Netherlands, Hart (102) assembled the data from 25 experiments when supplements, usually dried beet-pulp, were fed to dairy cows grazing abundant, good-quality grass. The amount of supplement fed ranged from 2.2 to 11 lb. per cow per day. There was a maximum increase in milk production of 4.4 lb. per cow daily. In one experiment, 8.8 lb. of dried beet slices reduced herbage consumption 19%, while increasing total dry matter intake. Similar small responses to supplementary feeding have been reported by Witt (308) and by MacLusky (162), who found that herbage consumption was reduced on poor, but not on good, quality pasture herbage. Hardison *et al.* (96) found that the feeding of 1 lb. of grain for each 6 lb. of milk resulted in a decrease of about 7.5% in grazing time. Burt (47) found that the feeding of 1 lb. of concentrates to 5 lb. of milk reduced daily grazing time by 1 hr. daily.

The experiments of Riddet and Campbell (236) show that New Zealand pastures do not allow full expression of the productive capacity of dairy cows, but it was emphasized that the use of concentrates to obtain maximum milk production from grazing cows may be uneconomical.

Corbett and Boyne (63) fed dried molasses sugar-beet pulp to dairy cows grazing abundant, intensively managed pastures in two trials (spring and summer). In the spring trial, the cows were fed 8 lb. of beet pulp per cow daily. The cows produced 38 lb. of milk per cow daily, which was 3.7% greater than the control cows. In the summer trial, 10 lb. of beet pulp were fed daily, and the milk yield of the supplemented cows was 11.3% greater than that of the control animals. Live weight was not materially affected. The herbage dry-matter consumption in the spring trial averaged 32.2 and 36.2 lb. per cow daily, for the supplemented and control cows, respectively. In the autumn trial, herbage dry-matter consumption for the supplemented and control cows was 27.0 and 30.6 lb. per cow daily, respectively. They conclude that when abundant good-quality herbage is available, it is uneconomical to feed supplements such as beet-pulp to grazing dairy cattle.

Stoddard *et al.* (270) and Arave *et al.* (8) supplemented irrigated pasture with 1 lb. of grain per cow daily, 1 lb. for each 10 lb. of milk, and 1 lb. for each 5 lb. of FCM milk. Milk production was 26.5, 30.9, and 31.6 lb. per day, respectively, in the first trial, and 37.1, 38.3, and 36.8 lb. per day in the second trial. The authors concluded that grain feeding was not profitable. According to Frens and Bosch (85), cows limited to 80% of the control acreage and fed dried pulp increased slightly in milk production. The concentrate supplementation was not economical.

The amount of pasture dry matter consumed when cows have limited grazing periods was discussed by McCullough (170). Reid *et al.* (229) were of the opinion that cows that grazed 2.3 lb/100 lb. of body weight in addition to 7 lb. of grain daily might have shown an increase in milk production with additional grain supplementation.

According to McCullough (169), feeding 7 lb. of hay or 5 lb. of grain daily reduced grazing time by 10%, ruminating time about 15%, but had no significant effect on milk production. He concluded that rumination time can not be used as an accurate predicator of animal performance. MacLusky (162) reported that the supplementation of pasture with 8 lb. of grain per day produced only 2.25 lb. of additional milk. Corbett (62) reported that the feeding of 8 to 10 lb. of concentrates per day, with various protein contents, to grazing cows failed to give an economical increase in milk production. A grain feeding table for cows on pasture was reported by Morrison (186).

METHODS OF USING PASTURE HERBAGE

The earliest method of using forage crops in summer was by open pastures. Eventually, fences came into use. Controlled grazing by means of tethered cows dates back to very early times. Rotational grazing is a relatively new system of using pastures, although the idea goes back into the sixteenth century (266). It involves dividing the land into two or more small enclosures and grazing each plot in turn. The Hohenheim system of pasture management combined intensive cultural practices with rotational grazing (184, 206). The progress in the use of rotational grazing largely has been due to the work of Riddet (235) of New Zealand and to the introduction of the electric fence (121).

Holmes (116) defined rotational management, whether the grass is grazed or conserved, as rapid grazing or cutting at the leafy stage, followed by a rest period, during which the grass regrows to a nutritious stage and replenishes its root system. According to Jones (137), good management of pasture means treating the plants in such a way as to enable them to provide the maximum quantity of leaves at the particular time the leaves are needed, thus preventing the plants from wasting their energy to produce the stems which no one wants. The art of retaining the plant in the leafy condition in June is to nip the little rudimentary seed heads in May that are destined to form the stem in June. Comparison between strains of grass and different methods of conservation needs to be done by reference to the animal itself; the actual production of meat and of milk per acre, rather than on S.E. and protein equivalent.

Rotational pastures. This subject has been reviewed by Holmes *et al.* (122) and by Shepherd *et al.* (258). According to Hodgson *et al.* (110), pasture yields, calculated as TDN, averaged 5,986 lb. per acre for a rotationally grazed pasture and 5,499 lb. for a similar pasture grazed continuously. This was an increase of 8.9% in favor of rotational grazing, but did not take into account the possibility that the forage from the rotational pasture was of higher biological value. Holdaway and Pratt (115), using bluegrass pasture, found no significant difference in TDN yield between rotational and continuous grazing.

Nevens (194), using heifers and milking cows, concluded that rotational grazing increased the yield and feeding value of pastures and also lengthened the pasture season. Nevens pointed out that pasture crops for dairy cattle should be rated on palatability, economy of production, timeliness of yield, length of pasture season, resistance to drought, amount of weeds, trampling, likelihood of bloat or other trouble, and adaptability to climate, as well as feeding value.

Brundage and Petersen (42) used four sets of identical dairy twins to compare rotational grazing with continuous grazing pasture management when no supplemental feed was fed. There was little difference in total or weekly animal production, in the form of growth, milk production, and maintenance from either management system. The area grazed under daily rotational system yielded nearly three times the production per acre as did that under continuous system. The authors concluded that when adequate good pasture and optimum weather conditions prevailed, daily rotational grazing did not seem to increase animal productivity over that obtained with continuous grazing, but did definitely increase the number of animals which could be maintained on a given area. Attention was directed to the necessity of close observation of both pasture and the animals, to insure adequate intake with minimum wastage in a system of daily rotational grazing.

In a comparison of continuous with rotational grazing, Harrison *et al.* (101) reported that the cows on the continuously grazed area produced more milk and consumed more grain than did those on the rotational area. The large amount of grain may have masked any difference between the two systems. Using the rotational system of pasturing heavy milk producing cows, followed by low producers, and then dry cows and young stock, Montague (184) concluded that proper management of the herd and pasture plots, combined with fertilizer treatment, is essential for the greatest net returns per acre in an intensive grassland system. McMeekan of New Zealand (176, 177) gave the following requirements essential to achieving high animal production from grassland: (a) A large amount of high-quality feed must be grown, the seasonal distribution must fit the animal requirement; (b) a large proportion of this herbage must be harvested by the animal, and (c) efficiency of conversion within the animal must be at a high level. The studies at Ruakura, New Zealand, fell into three main lines: (a) same animals, same stocking rates, but different grazing methods; (b) different animals, same stocking rate, and same grazing methods; and (c) same animals, different stocking rates, and same grazing methods. It will be noted that these types cover the three major grazing management factors under farm control.

The experiments at Ruakura were conducted on pastures yielding 8,000 to 12,000 lb. of dry matter per acre, virtually all-year pasture. A 10-yr. comparison of continuous grazing and rotation grazing with two herds of dairy cows was made. The only feed used was hay, for a 2-wk. period. The cows were milked at the same dairy with the same milkers. No cultivated crops were grown and no concentrates were fed. The cows on continuous grazing produced 266 lb. of butterfat per cow and 214 lb. per acre, whereas the cows on the rotational

grazing produced 324 lb. of butterfat per cow and 242 lb. per acre, annually. McMeekan speculated as to the possible reason for the small average difference in butterfat per acre, because of many pasture yield studies that have shown a marked superiority of rotational over continuous grazing in the amount of forage grown.

An experiment conducted to compare milk production of cows fastened in daily strips as opposed to intensive continuous grazing (187) resulted in no production differences. The importance of using animals capable of high milk production on continuous grazing was shown by the work of Wallace (296). The results of using cows of different levels of butterfat production on relative efficiency are shown in Table 6.

TABLE 6
Level of butterfat production and relative efficiency (296)

Butterfat per cow[a]	Butterfat per acre[b]	Efficiency
(lb.)		*(ratio)*
200	182	100
300	248	136
400	302	166

[a] Cows averaging 850 lb. of live weight.
[b] 10,000-lb. utilized dry matter per acre.

Van Horn *et al.* (288) reported data collected over a 5-yr. period with a forage mixture of orchardgrass, alfalfa, and Ladino clover under two systems of management: rotational grazing all season, and rotational grazing after the removal of one cutting for hay or silage. Rotational grazing which started after the removal of one cutting produced an average of 41% more calculated digestible nutrients, including both grazing and harvested forage, than continuous grazing all season.

Pratt and Davis (213), using brome-alfalfa-Ladino meadows, compared continuous and rotational grazing for three pasture seasons in Ohio. The cows were fed grain and hay in the barn. The rotational system consisted in moving the cows from one pasture to another. The pasture was then mowed and ensiled. The continuously grazed pasture remained unclipped. Under drought conditions, the accumulated forage from the rotational meadows was fed to maintain milk production. There was a 20% greater return of milk per acre from rotational grazing. In a Nevada experiment (104), cows were changed from an all-hay ration to pasture (3 to 4 wk.), then back to all-hay. Milk production (FCM) was increased 3.2 lb. per cow per day on pasture. When the cows were given hay as a supplement to pasture, milk production increased 3.7 lb. daily. McMeekan (178) reported very high yields of butterfat per acre from pastures using an intense system of rotational grazing, in which the cows were rotated among pastures several times a day.

Wilbur and Lundquist (304) reported that relatively high milk production may be maintained on good-to-excellent rotation pastures without supplementary feeds.

Lassiter *et al.* (156) summarized the results obtained with dairy heifers for a 13-yr. investigation to evaluate the continuous vs. the rotational system of grazing bluegrass pasture, and found no advantage of rotational grazing under their conditions. The yearly average production of TDN per acre by the continuous grazed pasture was 2,290 lb. and by the rotational grazed pasture, 2,284 lb.

According to Arnold *et al.* (12) and Camping *et al.* (48), a high output per cow can be achieved under a carefully managed, free-grazing system. The validity of the widespread assumption that a rotational system of management is essential for high milk production was questioned.

Strip grazing. Other systems of rotational grazing are called strip grazing, fold grazing, or ration grazing. MacLusky (162) defined fold grazing as a system in which an electric fence is used to allow cattle sufficient grazing for one day at each move. A back fence is used so that the cattle have access to only one part of the pasture at a time. Even without the use of a back fence, fold-grazing in practice improves animal-carrying capacity by enabling the farmer to gauge the grazing requirements of the herd. The surplus is conserved by reducing wastage from fouling and treading (more especially with long herbage), by making the animals graze more of the herbage by slightly reducing the amount of herbage available. This slight restriction of appetite does not impair milk production of cows yielding up to 40 lb. or more daily, provided the herbage offered is leafy and succulent. Fold grazing without close grazing is not an advantage in stock-carrying capacity.

Strip grazing is a general term which includes fold grazing, close grazing, and ration grazing (Holland), where the cows use a strip for a varying number of days. In ration grazing, the strips are grazed either once or twice daily Any of these systems may include the use of a back fence.

According to Holmes (116), strip grazing, when superimposed on rotational management, increases the utilization of herbage so that 20 to 40% more production per acre is obtained. In dry areas, continued strip grazing, three to six times over the same pasture in a season, results in serious fouling and reduced utilization.

The development of intensive pasture management systems has been comparatively recent. The Hohenheim system of pasture management combines intensive cultural practices with rotational grazing (206, 311). Holmes and Waite (121) proposed strip grazing (daily rotational grazing or close folding) with temporary electric fences. This system has been widely adopted in New Zealand and England. Several investigators (71, 102, 119, 215) reported 15 to 40% increases in milk yield from strip grazing as compared with rotational grazing.

The results of a comparison of strip and rotational grazed pastures with milking cows showed no significant differences in milk production (298). A comparison of daily grazing and four-day folding with low-producing cows showed no differences in milk yield, live weight change, or dry matter intake (125). In experiments where cows are fed above their production ability, the results are often misleading.

According to Brundage *et al.* (43–46), the importance of proper pasture management is magnified in Alaska by the brevity of the grazing season, the extended winter feeding period, and the limited amount of cleared land. These conditions emphasize the importance of obtaining maximum utilization of the available land, for increases in dairy farming often may be obtained more easily through an increase in the number of animal units maintained per acre than by an increase in farm size. A comparison was made of rotational grazing and strip grazing on bromegrass pasture. Strip grazing was only slightly more efficient than conventional rotational grazing. In this experiment, however, grain was fed, which may have covered up some of the effects of strip grazing. Arnold and Holmes (12) found no significant difference in milk production between strip and continuous grazing on nitrogen-fertilized pastures, compared with pastures receiving no nitrogen.

Brundage and Sweetman (43, 44) studied the correlation of herbage intake and milk production under rotational and strip grazing by using the combined Cr_2O_3-chromogen procedure to compute apparent dry matter intake and digestibility. Digestibility was a limiting factor under rotational grazing, but not under strip grazing. Shutt *et al.* (261, 262) called attention to the high protein content of immature forage obtained by rotational grazing. Ample and well-distributed rainfall was found to be a most important factor in the successful operation of the close-grazing scheme of pasture management. Svenstrup (273) compared rotational and strip grazing on 12 farms in Jutland and 12 farms in New Zealand, and found higher utilization of pasturage with strip grazing in both areas.

Brundage and Sweetman (45, 46) compared alfalfa–brome grass pasture under restricted grazing, and found that the cows utilized the pasture almost as efficiently as under rotational grazing. It was pointed out, however, that efficiency of utilization is a function of the potential producing ability of the grazing animals and the pasture being grazed. As the level of either or both rises, more intensive grazing may be necessary to maintain a comparable level of efficiency. Proctor *et al.* (215, 216) compared rotational grazing with close folding for milk production, using cows producing about 32 lb. of milk daily with no supplement. Close folding brought about 25% increase in milk production over rotational grazing. Better utilization under close grazing was due to less soiling of herbage. Dry cows can be made to eat the herbage following milking cows. Norman (199) reported the results of a comparison of close, lenient rotational, and continuous grazing with cattle. The resulting swards differed only slightly in agronomic quality. The lack of any advantage from rotational grazing was attributed to low levels of plant nutrients.

Campling *et al.* (48) compared strip grazing with nitrogen fertilizer, continuous grazing with nitrogen fertilizer, and continuous grazing with clover. The cows in the three groups produced about the same amount of milk (about 42 lb. daily) in trials conducted from May 1 to July 1. In the trial conducted between July 22 and September 22, the difference in milk production between the three groups did not appear to be significant at the lower level of milk pro-

duction (about 26 lb.). Arnold *et al.* (12) reported that wastage due to manure fouling for strip grazing and free grazing was 25 and 75%, respectively.

According to de Geus (71) of The Netherlands, ration grazing is an intensive system of utilizing grass. Cows are restricted by means of an electric fence to an area of pasture sufficient for one day's requirement. This necessitates moving the electric fence once a day. However, if the grass is abnormally long, two moves are made in one day. In continuous grazing, losses up to 40% occurred, compared with an average of 24% for ration grazing. At the Wisconsin Ashland Branch Station, the milking herd was divided into two groups: one group was rotationally grazed and the other group strip grazed (changed twice daily). The estimated TDN yields per acre for 1955 were 2,536 and 5,121 lb., and for 1956, 2,087 and 2,489 lb., respectively. The acres per cow for 1955 were 0.70 and 0.35, and for 1956, 1.10 and 0.65 (153).

A pasture was strip grazed and, although no supplemental feed was fed, the heifers gained 1.32 lb. daily, or 755 lb. per acre in body weight. Estimated TDN per acre was 5,227 lb. (165).

Soilage. Greater mechanization has made practical the system of cutting the forage and hauling it to the cattle. The early work on soilage was reviewed by Kildee *et al.* (144). The recent revival of soilage is due to mechanization which has reduced man-labor requirements. The freshly cut material is no longer fed on the ground but in forage racks or wagons with open sides. The terms zero pasture and green chop have been used. Since neither of these terms fits the bill, there is a tendency to revert to the old terms, soiling or soilage.

Graves *et al.* (89) obtained production data from a few cows receiving immature grass as pasture, soilage, hay and silage. The pounds of milk produced per acre were 4,575, 4,041, and 7,944 lb., respectively. The pasture group produced slightly more total milk than the soilage group, but the soilage cows produced 60% more milk per acre, according to Gullickson *et al.* (91). Henderson *et al.* (106) used milking cows to compare soilage with rotational grazing over a period of 3 yr. A grain mixture was fed at the rate of 1 to 8 lb. of milk daily during the 1st and 3rd yr., and 1 to 6 lb. during the 2nd yr., in an effort to prevent losses in milk production and body weight in both groups. The area required for 37 cows on soilage feeding was 32.9 acres as compared to 44.8 acres on pasture. The cows on pasture produced more total milk than the cows on soilage, but this difference was not significant. These results may have been due to the amount of grain fed and low milk-producing ability of the cows in both groups. Rotational grazing and soilage were compared, using six pairs of open heifers. No grain was fed and the results covered three, 2-yr. periods (106). The soilage-fed heifers gained 1.20 lb. and the pasture-fed heifers 1.55 lb. per day. The results appear to be in favor of pasture. Attention was called to the following points: the daily labor requirements for soilage practices will vary among farms because of field locations, crop yields, methods employed in harvesting the soiling crop, and herd size. Heavy rains not only make chopping difficult, but sometimes it is impossible to get necessary equipment into the fields. A field adjoining the barn should be available for grazing when the machinery

breaks down, or when heavy rains or other emergencies occur. There is also the problem of having a series of crops available to insure a constant supply of good-quality forage. Annual crops such as Sudan grass and millets do well in mid-summer, when many regular crops are in a relatively dormant stage due to lack of moisture and/or high temperature. Crops such as alfalfa, clover, brome grass, and orchardgrass that respond rapidly after cutting are essential. A hard-surface feeding platform is also essential to insure good sanitation. A cover or shade should be provided to prevent excessive moisture losses and to maintain palatability of the soilage.

The results of Henderson *et al.* (106) are not in agreement with those of Runcie (243), who found very little difference in the acreage needed per cow by grazing (0.87 acre for strip grazing) and soilage (0.77 acre for soilage). Runcie pointed out, however, that soilage enables the farmer to make better use of forage fields which, through distance or inaccessability, can not be used for grazing. Also, bloat is less likely to occur.

Bateman *et al.* (26, 29) compared pasture and soilage over a 2-yr. period. The results are shown in Table 7. Milk production per acre was about the same for 1957, but in 1958 the pasture cows produced 10,249 lb. and the soilage cows 9,109 lb. of milk per acre. The value above harvest costs in 1958 was $313 for grazing and $185 for soilage. The pasture season was 159 days and the soilage season was 136 days. It is necessary to allow plants to become more mature for soilage than for pasture. These data show the importance of proper grazing management in conducting comparative tests where grazing is involved.

A comparison of ten-day rotational, one-day strip grazing, and soilage was made by Smith and Keyes (265), using pasture forage, mostly grass. There was no significant difference in the amount of TDN produced per acre. The average feed costs of producing 100 lb. of milk by these three systems of management were $1.19, $1.29, and $1.55, respectively. There were no significant differences in the amount of milk per acre by the three systems of management. This may

TABLE 7

Production per acre from two methods of harvesting (26)

	Year	Graze	Green chop	Diff. green chop (+ or −)
Milk production 4% FCM *(lb.)*	1957	8,602	8,601	−1
	1958	10,249	9,109	−1,140
Butterfat production *(lb.)*	1957	349	345	−4
	1958	401	359	−42
Total man-hours	1957	11.8	29.0	+17.2
	1958	14.4	41.8	+27.4
Total machine-hours	1957	8.8	24.0	+15.2
	1958	4.6	30.9	+26.3
Harvest cost *(dollars)*	1957	.....	.....	.....
	1958	27.0	117.0	+90.0
Gross value per acre milk selling at $3.32 per cwt.	1957	286	286	0
	1958	340	302	−38
Gross value above harvest cost *(dollars)*	1957	.....	.....	.....
	1958	313	185	−128

have been due to the level of grain feeding, or to the limited milk-producing potential of the cows, or both.

According to Hoglund (113), an average of 3.03 acres per cow provided pasture and winter forage on farms where soilage was practiced during the grazing season, compared with 3.45 acres for farms where pasture was grazed in the usual way. In Minnesota (91), an average of 0.87 acre was required per cow when forages were green chopped compared with 1.45 acres when grazed. Production of milk was 2,528 lb. per acre for the green chopped forage and 1,525 lb. for the grazed pastures. It was estimated that 25 to 40% of the crop was wasted when pastures were grazed. More waste occurred with some grasses than with others.

Myers (191) visited farms where soilage was practiced, and concluded that green chopping (soilage) is best adapted to conditions where the size of the dairy enterprise is limited by land resources. Soilage may be adapted to some large farms where land is not a factor limiting the size of the dairy enterprise. Alternate uses for crop-land, such as production of high-value crops for sale, may be a more profitable use of resources than pasture and forage for dairy cattle. Myers (191) called attention to the advantages and disadvantages of the soilage system. Some of the advantages were: (a) Greater utilization of forage is possible than with rotation grazing or with continuous grazing, because of less loss from fouling due to droppings; (b) less energy is expended by cows in obtaining feed; and (c) damage to the soil and grasses through compaction is reduced. Some of the disadvantages of soilage were: (a) Additional costs are involved when the pasture crop is cut and brought to the cows; (b) a forage harvester, hauling, and feeding equipment are necessary, which means new capital investment if these implements are not already owned; (c) additional labor and power are required; (d) wet fields may make the daily job of cutting and hauling the feed difficult; and (e) the job may interfere with other work during peak labor periods. The additional costs may be offset by increases in income or by reduction of other costs. An increase in the size of the herd and greater sale of milk are possibilities, in some instances. Unusual conditions may justify soilage on some farms if pastures are located long distances from the barn, water is not available in the pasture, strip grazing makes fencing expensive, or the cost of grazing in the usual way is excessively high.

According to Hoglund (112), dairy farmers can decrease the acreage of pasture crops by as much as 25 to 30% by using soilage, but a farmer should consider the year-long feed requirements when he calculates the economic returns for different pasture systems. The extra forage saved might be used to feed the cows more forage during the pasture or barn-feeding periods, or it might be used to feed more cows. An individual farmer should consider whether he can afford to spend from 30 to 90 min. daily in chopping and hauling pasture crops to his cows. Will his labor earn him more when it is used on other enterprises? A dairy farmer should also consider whether the additional investment needed to shift to a soilage system might earn more money when invested in more or better cows, more fertilizers, or in a number of other alternatives.

By the use of a technique for determining pasture forage intake and digestibility, Reid *et al.* (228) compared alfalfa grazed conventionally with alfalfa fed as a soilage crop. The results in Table 8 show that the cattle, by selective grazing, consumed forage higher in protein and lower in fiber with a higher TDN content.

TABLE 8

Comparison of the composition and digestibility of alfalfa grazed and fed as a soiling crop (228)

Growth stage	Treatment	Composition of dry matter			Digestibility	
		Protein	Crude fiber	TDN	Protein	Crude fiber
		(%)				
Full bloom (1st growth)	Grazed	31.0	18.8	68	82	52
	Soiling	19.2	34.9	55	77	39
Post-bloom (1st growth)	Grazing	26.1	34.8	66	78	56
	Soiling	17.0	38.5	54	73	42
Vegetative (aftermath)	Grazed	27.7	15.3	73	81	57
	Soiling	19.4	27.3	64	77	52

In comparing relative values of soilage and pasture, the advantages and disadvantages should be weighed. The advantages of soilage are (a) no treading losses, (b) less fencing cost, and (c) no extra TDN required for barn maintenance. The advantages of pasture are: (a) increased nutritive value of immature grazed forage over soilage crop, (b) there is a grazing period before pasture plants yield enough to harvest, (c) higher costs of soilage, and (d) loss of fertilizing value of urine and manure when cows are kept in dry lot. Also, in the the comparison of soilage and pasture, the stocking rate is very important.

Stored feeding. Porter and Skaggs (211) used three groups of ten cows each to compare pasture, soilage, and silage. All cows were barn-fed 10 lb. of roughage daily to prevent bloat. No grain was fed below 17 lb. of milk daily for Jerseys and 24 lb. for Holstein cows. Forage yields harvested by machine were 100% greater than when the crop was grazed. The cows that grazed produced more milk for the amount of feed consumed than the cows fed the whole chopped plant, either as soilage or silage. Pasturing with dairy cows can save more than one-half the labor involved in harvesting the crop, even with the most modern machinery. This saving did not take into account the large investment, repair, and upkeep of the machinery, or the skilled labor required to operate the machinery. Machinery breakdown is a big item for ordinary dairy farmers. The results of this experiment clearly show the economy of grazing compared to machine-harvesting. Only where the dairy operation is on a large scale, and pasturing can not be practiced satisfactorily, does it appear that machine-harvested forage crops will be economical.

Owen *et al.* (201) compared rotational grazing, strip grazing, and silage, using Sudan grass with three groups of ten cows each. The average FCM per cow was 28.0, 26.6, and 29.0 lb., respectively, and the acres required by ten cows for 68 days were 6, 4.1, and 3.4, respectively.

Stone *et al.* (271), using milking cows, compared pasture (millet), soilage (millet), and two types of stored feeding (alfalfa hay and alfalfa silage). Grain

was fed with all rations. Milk production from silage and pasture was significantly less than from soilage and alfalfa hay. The silage ration was superior to pasture for milk production. A comparison of the forages for maintaining body weight ranked in the following order: alfalfa hay, silage, soilage and pasture.

Phillips and Baker (209) compared good pasture with barn feeding with three groups of cows. One group received pasture, another group was pastured but fed grain according to the Haecker Standard for milk production, and the other group was barn-fed the usual ration consisting of hay, corn, corn silage, and grain. The pasture cows were rotationally grazed when the forage was 8 to 10 in. high. Yields of pasture were determined by clipping cages. Milk production was the same for all three groups.

Larson *et al.* (153) and Larson (154) reported the results of an experiment at the Wisconsin Marshfield Branch Station, using three groups of cows. One group was fed hay and silage, a second soilage, and a third was strip grazed (changed twice a day). The estimated TDN yields per acre in 1955 were 4,514, 3,025, and 1,088 lb., and in 1956 were 3,519, 4,823, and 2,969 lb., respectively. The acres per cow for 1955 were 0.48, 0.82, and 1.16; and for 1956 were 0.47, 0.53, and 0.80. Larson (154) discussed a comparison of conventional grazing, rotational grazing, strip grazing, green feeding (soilage), and stored feeding based upon 3 yr. of study. Tall-growing forages of a predominantly alfalfa and grass mixture were used. The losses of forage were 60-70, 43, 31, 2, and 11%, respectively. The milk production per acre was greater for both soilage and stored feeding than for the various systems of grazing. According to Larson, the advantages of stored feeding are: (a) forage can be harvested at the same time, at near the same stage of growth, with the smallest amount of field losses, (b) eliminates most fences, (c) prevents summer drop in milk production, (d) no losses of forage due to trampling, (e) permits closer observation of cattle, (f) less bloat, (g) easier to supply water to the herd, and (h) adapted for use with any sized herd. The disadvantages are: (a) need dry lot near the barn, (b) sanitation may become a problem, (c) dairymen are not freed from winter routine during the summer, and (d) topography of an area, or a single farming unit, is such that grazing is the only practical method of using the forage.

The large losses of forage associated with grazing as reported by Gullickson *et al.* (91) and Larson (154) may have been due to improper grazing management, as suggested by Porter and Skaggs (211). Also, the data reported by Bateman (26), as shown in Table 7, indicate that when tall crops (alfalfa in mixture) are properly grazed, milk production per acre is at least equal to soilage. The works of both Porter and Skaggs and Bateman were conducted under irrigation and, as has been pointed out, there is less loss of pasture forage by fouling when plenty of moisture is available.

In comparing the value of pasture with harvested forages, such as soilage, hay and silage, it is well to keep in mind that cattle use the top of the crop and not both ends of the plants while grazing. The investigations of Hardison *et al.* (97) indicate that alfalfa stems are only 87% as digestible as the tops. It is interesting to note that as the trial advanced the stems decreased about 0.6

of a percentage point per day in digestibility. On the other hand, there was no marked decrease in the digestibility of the top part of the forage as the trial advanced. In using soilage and stored forage, it is not only necessary to haul the stems out of the field but is also necessary to haul most of them back. As has been previously pointed out, the manure problem in the use of both silage and stored crops should be considered in deciding which system of forage utilization should be used.

Pasture for young stock. The calf is born with a four-compartment stomach, but only the abomasum is functional. The age at which transition to the ruminant occurs, from the standpoint of the relative size of the rumen and fiber digestion, depends on the diet (24). Given a limited quantity of milk, the calf will eat alternative feeds at a very early age, provided they are palatable. The trend is to feed the calf so that it becomes a ruminant at an early age, in order to take advantage of the microbial digestion and synthesis of high-quality protein and vitamins. Also, as the rumen starts to function, the incidence of calf scours is considerably reduced. Browlee (41) and Warner *et al.* (300) showed that roughage increased rumen volume, and that a diet of milk and concentrates increased greater development of rumen papillae than a diet of milk and grass. This, in turn, gave more development than milk and hay.

The development of the microbial population in the rumen of the very young calf was reviewed by Roy (240). The protozoan population can be established in the rumen, once the pH is stabilized near neutrality. This occurs at about 8 wk. of age. The beneficial action of the tetracyclines in increasing appetite and efficiency of food utilization during the transition to the ruminant may be due, in part, to their effect in raising the pH of the rumen contents, as shown by Mann *et al.* (163). Roy and Balch (241) found that a calf as early as four days of age showed practically normal rumen contractions and attempted to ruminate.

The widespread belief exists that pasture is of little value to the young calf before 6 mo. of age. McMeekan (175) and Roy *et al.* (242) have shown that calves start eating grass from about the seventh day onwards and can be entirely dependent on good-quality pasture grass at 8 wk. of age.

Experiments at the Ruakura Research Station (5) reported the following findings: (a) calves start to ruminate as early as seven days and rarely later than 3 wk.; (b) by 3 mo. of age the calf has a rumen as large as that of an adult cow, in relation to its body weight, and becomes relatively larger at 5 mo.; (c) calves fed grass in addition to milk show better rumen development than milk-fed calves; (d) early-weaned calves (8 wk.) subsequently show more rapid development of the rumen than late-weaned calves (18 wk.) and are able to handle pasture at the same age; (e) after early weaning (8 wk.), calves require 5 to 7 hr. grazing per day on high-quality pasture to obtain sufficient nutrients to grow at a reasonable rate; (f) on poor-quality, fibrous pastures the calves have to graze longer, waste time in searching for suitable herbage, and ruminate longer in an attempt to satisfy their needs; and (g) calves are highly selective grazers and tend to starve themselves on unsuitable pastures.

Several investigators studied the ability of young calves to digest immature pasture grass. Conrad *et al.* (61) conducted two, seven-day digestion trials on 9-wk.-old calves. The calves digested 71.5 to 73.5% of the dry matter and 70.5 to 82.9% of the cellulose. Armstrong *et al.* (14) fed 10- to 12-wk.-old calves fresh grass (preflower) that had been in cold storage, and obtained coefficients of digestibility for organic matter, crude fiber, NFE, and cellulose of 77, 80, 83, and 84, respectively. The crude protein digestibility, however, was only 60%, which is on the low side. Preston *et al.* (214) found that 3-wk.-old calves digest 75% of the dry matter and 84% of the cellulose of young grass. The forage was mostly grasses, with a small amount of clover and weeds. The digestibility trial covered the 3rd through the 10th wk. of age. McArthur (167), using cows and 8- to 10-wk.-old calves, found that the coefficients of digestibility for dry matter and crude fiber were about the same (80% for crude fiber).

Roy *et al.* (242) reported that calves on pasture were fed 450 lb. of whole milk during the first 8 wk. of life. From the 5th wk. on, the amount of milk was gradually reduced and replaced by an equal volume of water, until the milk was entirely eliminated at 8 wk. of age. During the milk-feeding period, dried grass was provided, but little was eaten. In general, calves preferred pasture grass to the artificially dried product. After weaning at 8 wk., the calves were entirely dependent on pasture grass, except that wheat straw was available. During the spring flush of grass, all of the calves were fed a small quantity of the straw each day. The calves grew normally throughout the spring and early summer, but because of the deterioration of the pasture in late summer it was necessary to start supplemental feeding in the middle of August (England). Five to seven pounds of dried grass were fed per calf daily as a supplement. Most of the calves began to eat grass before they were 3 wk. old.

McCullough and Sell (172) pointed out the possible advantages of raising dairy calves on pasture: (a) reduces labor required to clean calf-barn, (b) decreases sickness and death losses due to improperly cleaned calf-barns, and (c) reduces feed cost. In their study, the newborn calves were left with their dam for 48 hr. From the third through the sixth day, the calves were confined to a small pen, where they were tattooed, dehorned, and taught to drink from a nipple pail. On the seventh day, the calves were placed on pasture. Whole milk was fed for 6 wk. at the rate of 10% of birth weight. The calves were rotated on pasture each week to allow forage regrowth. In experiments where calves were fed grain, it was offered as soon as the calf would nibble at it—usually at about 14 days of age. These investigators emphasized the need for high-quality forage at all times. They also recommended keeping the forage in a leafy stage by frequent mowing and top dressing (forage should be kept under 10 in. in height), dividing the pasture area into small lots, and rotating at 14-day intervals. If internal parasites are a problem, place calves on pasture at 1 wk. of age and continue milk feeding for 6 wk. If grain is to be fed, commence grain feeding at 2 wk. of age. Do not attempt to grow calves on poor pasture, but provide shade, water, salt, and adequate feed at all times.

Conrad and Hibbs (60) placed one group of calves on Ladino clover pasture at 13 to 26 wk. of age and compared them with barn-fed calves on a 2:1 ratio of hay to grain. The calves fed Ladino clover pasture and 2 lb. of oats daily gained more rapidly from 18 to 26 wk. than barn-fed calves. The results indicate that good legume pasture may be substituted for hay in the high roughage system after calves are 3 mo. of age.

Stillions *et al.* (269) compared barn feeding, alfalfa-Ladino-brome pasture alone, and the same pasture with hay free choice. The calves were placed on pasture at eight to 14 days of age and limited whole milk was fed to 49 days of age. The calves consumed an average of 64.5, 43.4, and 36.9 lb. of grain, and 44.5, 0.0, and 10.1 lb. of hay, respectively, during the first 60 days. The average daily gains were 0.97, 1.14, and 1.10 lb., respectively. These differences were not significant. From 60 to 116 days of age, the calves consumed an average of 187.3, 110.0, and 107.9 lb. of grain and 256.2, 0.0, and 20.5 lb. of hay, with an average daily gain of 1.46, 1.67, and 1.70 lb., respectively. The differences in gains were not significant. Fecal egg counts were negative. Feeding hay with pasture appeared to improve fecal firmness and the general appearance of the young calves.

The parasite problem is serious in the Southern States. Grinnells (90) stated that calves should not go to pasture until 10 mo. of age. When internal parasites are not a problem, thrifty calves do well on pasture after they are 2 mo. of age if they are supplied with supplementary feed, shade, and fresh water. McCullough (172) found that calves raised by the calf starter method did well when placed on good pasture at seven days of age with no hay. Rust *et al.* (244) placed 3- to 4-mo.-old calves on good bluegrass pasture. One group received 3 lb. of grain per calf per day and gained an average of 1.2 lb. daily through the summer; whereas, the group that received no grain gained 1.0 lb. daily for the same period. The average dry matter intake from pasture was 2.6 lb. per 100 lb. of body weight for the group receiving grain, as compared to 3.7 lb. for the group receiving no grain. The coefficients of digestibility of the dry matter and protein of pasture herbage were 64.5 and 60.6%, respectively. In general, the grain-fed group had a lower level of parasite infection than the no-grain group.

In the past, high-quality forage in the form of pasture was seldom available for heifers and cows. In evaluating the early work, it is well to keep this in mind. Bender and Bartlett (30) observed losses in weight of 40 to 100 lb. per head during the first months after fat 2-yr.-old heifers were turned on pasture; whereas, heifers winter-fed on roughage suffered no loss in weight and made greater total gains for the season. According to Hayden (103), 29 Jersey heifers, 1 to 2 yr. of age, kept on pasture for 159 days, made daily gains for the year equivalent to 0.8 lb. per head; whereas, 22 Holstein heifers of the same age, pastured 151 days, made daily gains during the year amounting to 1.0 lb. per head. Henderson and Anthony (107) reported that daily gains of yearling dairy heifers kept on pasture for 194 to 203 days ranged from 0.3 to 1.13 lb. per head. Heifers kept on pasture as the only feed gained 0.7 lb. for the Brown Swiss and up to 0.96 lb. for Ayrshire heifers daily.

Reid *et al.* (229) used six pastures at three stages of growth to determine the amount of nutrients consumed by growing steers. The results showed that free-grazing steers weighing from 500 to 600 lb. consumed sufficient digestible protein and TDN to meet their needs for growth without supplementation. Severely restricted intakes of protein and/or TDN resulted in reduced gains in body weight.

DISORDERS OF DAIRY CATTLE DURING THE SUMMER

Cows on pasture may be affected with bloat, or poisoned by eating plants that contain toxic levels of hydrocyanic acid.

Bloat. One of the problems arising from using improved pastures as feed for dairy cattle is that of bloat. Normally, cattle have an efficient eructation mechanism and are able to belch several times as much gas as is ordinarily produced. The subject of bloat has been reviewed previously (56, 57, 74, 132, 203); consequently, this review will be limited to some of the recent papers dealing with pasture bloat.

It is difficult to obtain estimates of the loss of dairy cattle due to pasture bloat, since surveys on the incidence of bloat and deaths include all classes of cattle and all kinds of bloat. Nichols (196) reported that a survey of over 8,000 farms in Wisconsin showed that about 30,000 cows bloated each year; about 6,000 died, which represented a loss of more than $2,000,000 in Wisconsin alone. An extensive survey conducted by the New Zealand Dairy Board in 1953 and 1954 (131) indicated that of the 4,943 farmers who provided information, about 12% considered bloat to be a serious problem in their herds. One dairy cow in every 200 died from bloat during that period in New Zealand. This loss, however, does not take into consideration the loss in milk production which is due chiefly to the decrease in feed intake. This was shown in the average dry matter intake recorded for the various degrees of bloat in stall-fed identical twins by Johns (131). Another loss is due to the fear of bloat, which results in growing less productive pastures.

Bloat may originate in the animal or in the herbage but, in either case, the eructation process fails. The gas produced in the reticulo-rumen collects as a pocket in the dorsal rumen at a point higher than the cardia, the entrance to the esophagus. The cardia is normally below the level of the ingesta. Eructation has to be a coordinated process involving activity of the reticulum, rumen, and esophagus.

The cause or causes of bloat. The cause of this malady is not known, but there are many theories which attempt to explain this condition, such as too much gas production, paralysis of the rumen musculature, the physical nature of feed, and foaming or surface tension. These theories have been described by several investigators and will not be discussed in this review.

Pasture management. As was pointed out by Johns (132), the pasture should not be managed so that mature grass is dominant. This procedure may result in decreased nutritive value of the forage. Dougherty (74) pointed out that pastures containing less than 50% legumes may be considered safe. According

to Johns (132), safe proportions will always be difficult to define, since they will depend on the potency of the legume, the appetizing quality of the mixture, and the selective grazing habits of the cattle. Also, the cost of establishing and maintaining legume grass mixtures or straight grass pasture may be high. Troughton (281) produced bloat in grazing trials with pastures of less than 33% clover under New Zealand conditions.

Controlled grazing includes a restriction of legumes, especially the succulent tops which are believed to be the most potent part of the plant, and an alteration in the grazing method to reduce the rate of intake (132). Control of selection has been accomplished by the use of soilage, but this practice does not necessarily insure freedom from bloat.

The elimination of selective grazing by strip grazing to make the cattle consume the whole plant has been suggested. As was pointed out by Reid (225), this method may reduce the incidence of bloat, but can not give full control of severe bloat without reduction of forage intake and a consequent loss of milk production. This system has a higher labor cost and may lead to overgrazing.

Feeding of roughage. The effectiveness of Sudan grass hay and Sudan pasture in the prevention of acute bloat in cattle was reviewed by Cole and associates (57). Johns (131) failed to prevent acute legume bloat in dairy cattle prefed up to 2 lb. of hay per animal. Hogg and Barrentine (111) prefed 5 lb. of hay to cattle before they were grazed on Ladino clover and found a reduction in bloat in the morning but not in the afternoon. Recently, Colvin *et al.* (59) found that the over-night feeding of an average of 12 lb. of oat hay per cow significantly reduced the incidence and severity of acute bloat in dry cows when pastured on succulent, immature alfalfa.

Antifoaming agents. Authorities are not agreed on the relative occurrence of frothy and free gas pasture bloat. In frothy bloat, the degree of bloat appears to be associated with the stability of the foam. In general, antifoaming agents act by displacing the foaming substance from the surface of the foaming liquid and by replacing it with a nonfoaming surface layer. The feeding of methyl silicone (a good defoamer in vitro) as a bloat preventative does not appear to be effective, according to Johns (132) and Hungate *et al.* (128).

The most effective antifoaming agents thus far developed appear to be the emulsified vegetable oils. These may be given as a drench, added to the drinking water, or applied to the pasture as a spray. Emulsified peanut oil is applied at the rate of 2 oz. per cow daily. Bloat was also prevented on clover pasture by spraying with tallow. Whale oil, medicinal paraffin, vegetable turpentine, and a richinoleic acid derivative were effective in the prevention of bloat, but some of these products tainted the milk (173, 174).

The mixing of either soybean oil or lard oil with lecithin greatly reduced the incidence of bloat when mixed with the grain fed to the animals before being turned to pasture (134). Bloat occurred among cattle pastured on land that had been fertilized with phosphate. The addition of 0.25 lb. of soybean oil per feeding in a grain mixture gave good control for several hours, but ground soybeans (oil content approximately 0.25 lb. per feed) failed to reduce bloat.

Lard oil (100–200 ml.), administered by stomach tube, gave prompt relief in severe cases of bloat (39). Johnson *et al.* (134) and Brown *et al.* (39) added 1 to 2% of water-dispersible oil to the drinking water and reduced the incidence and severity of pasture-type bloat.

Reid (225) prefers the use of oil sprayed on the pasture, rather than the addition of oil to drinking water. It is necessary to maintain an effective level of oil in the rumen throughout the time the animal is grazing a dangerous pasture. It was early found that a limiting factor in the case of oils and fats is the rapidity with which they are lost from the rumen by passing down the digestive tract. Good bloat control was achieved by spraying oils on pastures, but this is only an emergency measure. Its greatest values lie in making it possible for cattle to remain in an otherwise dangerous pasture. Antifoaming agents are effective only against frothy bloat. Spraying is laborious.

Penicillin for bloat control. Barrentine *et al.* (23, 24) controlled bloat in steers for one and one-half to three days by feeding 50 to 75 mg. of procaine penicillin or its equivalent as potassium penicillin. They also obtained practical control of bloat by the free-choice feeding of salt, which contained 800 mg. procaine penicillin per pound. According to Johns (133), the use of 100,000 to 200,000 units of penicillin per cow gave good control in the spring (about 50% effective). No penicillin passed into the milk when it was fed. According to Emery *et al.* (81), the incidence of bloat was reduced about two-thirds by feeding 100 mg. procaine penicillin per cow per day, either with the grain or in the salt on a free-choice basis. The efficiency of treatment appeared to decrease as the season progressed. An average incidence of 9.7% bloat, with a mortality of 4.2% of the bloated animals, was found for 739 cows over a 150-day pasture season. Thomas (277) observed no severe bloating in cattle for 12 to 96 hr. after the administration of 100 mg. of procaine penicillin per animal. Control animals showed extensive bloat. Brown *et al.* (39) and Johnson *et al.* (134) confined dairy cattle to a dry lot and fed fresh alfalfa soilage ad libitum. The oral administration of 75 mg. of procaine penicillin per animal daily appeared to reduce bloat when first given to steers. Later in the season, when the bloat-producing potential of the forage increased sharply, there was just as high an incidence of severe bloat in the penicillin-fed group as in the controls.

There is good agreement among investigators that the feeding of penicillin prevents pasture bloat for a variable time after the start of the regime, but its effectiveness becomes less with time. The work of Smith and Emery (264) indicates that penicillin fed to cattle remains in the rumen longer the first day of feeding than after 3 wk. of feeding. These results suggest that a penicillin-destroying principle increases with time and may explain the ineffectiveness of this antibiotic as a bloat preventative with continued administration.

Prussic Acid (HCN) poisoning. The toxicology of cyanogenetic glycosides was reviewed by Kingsbury (145) and Franzke (84). According to vander Walt (287), poisoning of ruminants by cyanogenetic plants depends upon the amount of the plant consumed, the previous diet of the animal, the hydrogen ion concentration of the stomach contents, the percentage of total HCN present

in the free state in the plant, the concentration of cyanide-liberating enzymes present in the plant, and the HCN content of the plant. According to Moran (185), the intact glycoside is not toxic. The toxicity of the compound is caused by hydrolysis which liberates a free HCN molecule. There is little free HCN in healthy, active growing plants (liberated by enzyme). Ruminants are more susceptible to HCN poisoning than are nonruminants. Plants which contain more than 20 mg. % of HCN can be considered toxic. Boyd *et al.* (33) gave the safe limits of cyanide content as shown in Table 9.

TABLE 9
Tolerance to various levels of HCN in forage (33)

HCN	Relative degree of toxicity
(mg/100 g. dry tissue)	
0– 25	Safe to pasture
25– 50	Safe to pasture
50– 75	Doubtful
75–100	Dangerous to pasture
More than 100	Very dangerous

Young Sudan grass (4 in. high) may be high in HCN, but it declines as the plant matures until 16 in. high, when it is safe to pasture. This is the basis for the recommendation not to pasture Sudan grass until belly-high to the cow (33). Contrary to common opinion, frost does not increase the HCN content of Sudan grass. Boyd *et al.* (33) showed that drought did not increase the cyanide content of Sudan grass, which is also contrary to common opinion. The many toxic weeds that sometimes affect cattle have been reviewed by Kingsbury (145).

EFFECT OF PASTURE AND SOILAGE ON MILK FLAVOR

Desirable flavors. In order to continue marketing milk and milk products, it is necessary to maintain and stabilize the flavor that appeals to the consumer. This is of greater importance because of the trend to hold milk longer between the time milk comes from the cow and its consumption. This review will cover the effects of pasture and green feed on the flavor of milk. Several reviews have been concerned with flavors of milk products from a general point of view. Kende (142) concluded that green feed contains some substance or substances which, when fed to the cow, protect milk against certain off-flavors. Since that time, numerous workers have observed the difference in susceptibility of winter and summer milk to oxidized flavor. Brown *et al.* (40) reported that the feeding of dry feed increased the tendency for the oxidized flavor to develop in milk and that fresh pasture decreased this tendency.

Dahle and Palmer (66) found that spontaneous oxidized flavor of cow's milk was prevented by feeding pasture—green alfalfa and clover. Hening and Dahlberg (108) found that the occurrence of oxidized flavor in milk was much less in the summer months than in winter months, but there was no definite correlation between the exact period of feeding green legumes and the occurrence

of the off-flavor in the milk. Greenbank (88) reported that the development of the oxidized flavor in milk was not always inhibited by feeding green feed. Beneficial effects, however, were indicated by the fact that in every case there was a decrease in the intensity of the oxidized flavor by the end of the 5th wk. on pasture. Garrett *et al.* (87) concluded that alfalfa silage is almost equal to spring pasture in putting yellow color in milk, and is equal to or better than pasture in producing milk of fine flavor and high resistance to oxidized flavor. According to Thurston (278), cows on abundant pasture produce milk that is not susceptible to oxidized flavor, whereas the milk from the same cows on dry feed is susceptible.

Krukovsky *et al.* (149, 150) reviewed the literature, which showed that the carotene, vitamin A, and tocopherol contents of milk fat vary with the type and quality of the roughage fed. A relationship was also found between the tocopherol content of milk fat and the ability of milk to resist reactions that produce oxidized flavors.

According to Krukovsky, the susceptibility of milk to oxidized flavors is affected not only by the antioxidant activity of the fat (tocopherols) but also by the presence and availability of readily oxidizable nonflavor-forming compounds in the plasma phase of the milk. These properties of milk are influenced by the type and quality of roughage fed. Cows that were pastured on predominantly orchardgrass with some Ladino clover, green oats, and alfalfa-brome-Ladino clover produced milk stable to oxidized flavor. Milk from cows pastured on orchard grass was exceptionally stable toward oxidized flavor, whereas milk from cows on alfalfa-bromegrass-Ladino pasture was the least stable of the kinds of pasture tested. Hvidsten *et al.* (126) found that milk produced on pasture is more stable than milk produced in the preceding barn-feeding period.

Undesirable flavors. Good-quality pasture, under certain conditions, may impart undesirable flavors and odors to milk. Babcock (17) found that the feeding of green alfalfa at the rate of 30 lb. per cow 1 hr. before milking produced pronounced off-flavors and odors in the milk. When reduced to 15 lb., the off-flavors and odors were still present. When using alfalfa pasture, the cows should be removed from the pasture 4 or 5 hr. before milking. Roadhouse and Henderson (239) found that feed flavor appeared in the milk 20 min. after the ingestion of alfalfa juice, or following the feeding of green alfalfa or alfalfa hay. These investigators (238) also found that the feeding of green alfalfa or alfalfa hay 1 to 2 hr. before milking produced strong, undesirable feed flavors and odors in the milk. As the intervals between feeding and milking increased, the intensity of the feed flavors decreased. A 5-hr. interval between feeding and milking eliminated the feed flavor in the milk. A distinct and undesirable feed flavor resulted from feeding 5 lb. of alfalfa hay and 10 lb. of green alfalfa 1 to 2 hr. before milking (239).

Davis (70) presented a list of weeds that are sometimes found in pastures and the off-flavors they produce in milk. He also stated that weed taints are naturally more frequent when pasture is scarce. Certain cows may show a desire for certain weeds even when pastures are abundant. Aurand and Moore (16)

reported that three different mineral supplements, advertised to prevent weed flavor in milk, failed to do the job on onion-infested pasture. Wylie (314) reported that the most practical method of onion-flavor control in milk was pasture management.

The feeding of 15 lb. of green rye 1 hr. before milking produced only slightly abnormal flavors in the milk (18). The feeding of 30 lb. of green rye 1 hr. before milking produced a small increase in abnormal flavor. Rye pasture gave more offensive odor than wheat pasture (6). Removing the cows from pasture 4 hr. before milking eliminated or greatly reduced the off-flavors. Trout and Horwood (282) indicate that Balbo-rye pasture does not have the adverse effect on the flavor of milk that is produced by common rye pasture. The methods of avoiding or correcting off-flavors in milk include: careful herd management, feeding an adequate ration, and keeping cows off the weedy pastures 3 to 8 hr. before milking (72).

According to Roadhouse (238), the feeding of 25 lb. of green-cut alfalfa gave a distinct feed flavor to milk, but the off-flavor was most pronounced when the feed was placed before the cows 2 hr. before milking. The effect of grazing cows on an alfalfa-bromegrass pasture was investigated by Trout *et al.* (283). Intense off-flavor was found when the cows were grazing heavily. Alfalfa contributed more to the off-flavor than did the bromegrass. When the cows were kept off the pasture 7 hr. prior to milking, no feed flavors were noticeable in the milk.

Flavor scores showed no clover flavor to be present in the milk produced by cows grazing on a Ladino clover, fescue, and orchardgrass pasture up to 2 hr. previous to milking (259). Abnormal flavor and odor were produced in milk by feeding quantities of rape up to 30 lb. per cow per day 1 hr. before milking (19). When fed immediately after milking, rape had practically no effect on either the flavor or odor of the milk. Milk production was not affected when the dairy herd was taken off pastures for 3 hr. before the afternoon milking (67). Marryatt (164) found that feed flavor in milk was much reduced by keeping the cows in dry lots for 3 hr. before milking.

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SOME EFFECTS OF FEEDING VARIOUS FILLED MILKS TO DAIRY CALVES. I. PHYSICAL CONDITION AND WEIGHT GAINS, WITH SPECIAL REFERENCE TO LOW-FAT RATIONS [1]

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SUMMARY

Various filled milks and low-fat rations were studied in an attempt to determine some of the factors responsible for the unsatisfactory gains, poor physical condition, and deaths which have been reported as occurring in calves fed rations containing highly unsaturated vegetable fat. Under the conditions of the experiment, the results of feeding corn oil filled milk to dairy calves were as follows: (a) animals appeared emaciated and unthrifty, (b) a marked diarrhea was observed, (c) an increased susceptibility to pneumonia and a high mortality rate occurred when infection was not rigidly controlled, (d) symptoms of anorexia and muscular involvement were noted, (e) poor feed utilization and unsatisfactory weight gains were obtained.

Calves fed hydrogenated vegetable oil, lard, or butter oil filled milks and whole milk were not adversely affected in the above manner, except as follows: Animals receiving lard and butter oil, prepared from oxidized butter, also exhibited symptoms of muscular involvement. Supplementation of the corn oil and lard filled milk diets with high levels of tocopherol prevented the development of muscular involvement, but did not improve weight gains of calves fed corn oil. Weight gain and feed utilization data obtained on the various rations suggest that the inclusion of corn oil in the diet depresses growth in some manner.

Diets containing as low as 0.10–0.15% butterfat were not detrimental to growth and physical condition of calves under the experimental conditions. A number of the animals fed several of the filled milk and low-fat rations were satisfactorily maintained on the liquid diets to 5-6 mo. of age.

Previous studies (7, 8, 9, 11, 13) have established that highly unsaturated vegetable oils adversely affect growth and physical condition when used as substitutes for butterfat in the diet of dairy calves. This research represents an attempt to determine some of the factors responsible for the unsatisfactory results obtained with highly unsaturated vegetable oils. Data pertaining to physical condition, appearance and weight gains of calves fed various filled milks and low-fat rations are presented in this paper. Those relating to digestibility, blood, urine, necropsy, electrocardiographic, and other studies will be reported later.

EXPERIMENTAL PROCEDURE

Approximately 150 calves were involved in this study, which extended over a period of 4 yr. The animals used in this experiment were, with several excep-

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tions, purebred or grade Holstein bull calves. All animals received colostrum for from two to three days following birth and were subsequently fed whole milk until they were placed on an experimental ration at approximately 14 days of age. Whenever possible, the calves were maintained on the experimental diets until 90 days of age. Although all trials were not run concurrently, environmental conditions were maintained as nearly uniform as possible for all subjects.

Each calf was weighed at weekly intervals. Frequent observations were made and recorded in regard to the physical condition and other pertinent data concerning each animal. Regarding health supervision, the initial group of calves receiving the corn oil filled milk were treated only when symptoms of respiratory infection were noted, or the subjects were afflicted with infectious scours. All subsequent animals on the corn oil filled milk and other diets were treated with therapeutic doses of penicillin in oil or terramycin in sterile distilled water. These antibiotics were administered intramuscularly, whenever the daily rectal temperature of the calf reached 103.5° F. or greater.

All of the fats and oils studied were refined or fit for human consumption. The filled milks were prepared by homogenizing the various fats and oils into pasteurized skimmilk at a rate calculated to provide a product containing 3.5% fat. Each filled milk was heated to approximately 65° C., then passed twice through a Gaulin two-stage homogenizer at 2,500-lb. pressure. All diets were prepared weekly, except as otherwise noted, and stored at 5° C.

Vitamin A was provided at the rate of 160 I.U. per pound of body weight, whereas vitamin D was fed at the rate of 500 I.U. per 100 lb. of body weight. Both vitamins A (cod liver oil concentrate) and D (Viosterol) were added prior to homogenization. All rations except the whole milk diet were so supplemented with vitamins A and D. Animals fed whole milk were provided only with vitamin D in capsule form. The calves on all diets received the following amounts of trace minerals in elemental form: Fe 70, Cu 7, Mn 10, Co 5, I 1.5, and Zn 0.9 mg. per 100 lb. of body weight. A solution of these minerals was added to the rations. Anhydrous magnesium carbonate was added to each diet immediately prior to feeding at the rate of 5 g. per 100 lb. of body weight daily. A mixture of equal parts of steamed bone meal and salt was fed free-choice.

The whole milk diet consisted of herd milk which was standardized at 3.5% butterfat and subsequently pasteurized and homogenized. The fortified skimmilk ration was prepared by adding sufficient nonfat dried milk solids to pasteurized skimmilk, so as to provide a product which was isocaloric, with the filled milks containing 3.5% fat. This diet contained approximately 0.15% butterfat. The skimmilk ration tested 0.1% butterfat and did not contain any additional nonfat dried milk solids. The fortified skimmilk plus corn oil diet was identical to the fortified skimmilk ration, with the exception that sufficient corn oil was incorporated to provide a product with approximately 3.5% fat. The fortified skimmilk plus hydrogenated vegetable oil ration was prepared in the same manner, except that a blend of hydrogenated cottonseed and soybean oils was used as the source of fat. This type of blend is frequently used in frozen desserts.

A mixture of B-complex vitamins and liver residue was administered orally to provide the various ingredients at the rates indicated in Table 1. As detailed

TABLE 1
B-complex and liver residue supplementation

Ingredient	Mg/100 lb. body weight daily
Thiamine chloride	3.00
Riboflavin	6.50
Pyridoxine	3.00
Calcium pantothenate	15.00
Inositol	100.00
Choline chloride	1,000.00
Menadione	1.07
Nicotinic Acid	15.00
p-Amino Benzoic Acid	10.00
Folic Acid	5.00
Biotin	0.05
B_{12}—0.1% trituration	0.05
Liver residue (20%)	20.00

in a later report, several methods of tocopherol administration as well as tocopherol supplements were investigated. In each instance an equivalent of 500 mg. of DL alpha tocopherol or alpha tocopheryl acetate was administered daily to each calf. While only certain groups of corn oil and lard filled milk animals received tocopherol supplementation, all calves on the skimmilk and fortified skimmilk plus corn oil rations received such supplementation.

The filled milks or other diets as supplemented with mineral and vitamins constituted the sole source of nutrients, except as noted otherwise. With the exception of the skimmilk and the fortified skimmilk plus added fat diets, all rations were fed at levels calculated to provide total digestible nutrients according to the recommendations of the National Research Council Committee on Animal Nutrition (12).

RESULTS AND DISCUSSION

A description and an enumeration of the various diets studied in this phase of the research may be found in Tables 2 and 3, respectively.

TABLE 2
Major components and estimated TDN content of diets

Diet	Lb. skimmilk	Lb. added nonfat dried milk solids	Lb. added fat	Estimated TDN content[a]
	(per 100 lb.)			
Skimmilk	100.0	....	....	8.7
Corn oil filled milk	96.5	....	3.5	15.5
Hydrogenated corn oil filled milk	96.5	....	3.5	15.5
Lard filled milk	96.5	....	3.5	15.5
Hydrogenated cottonseed and soybean oil blend filled milk	96.5	....	3.5	15.5
Whole milk	.....	....	....	16.3
Butter oil filled milk	96.5	....	3.5	15.5
Fortified skimmilk	90.5	9.5	....	15.5
Fortified skimmilk plus corn oil	87.3	9.2	3.5	22.0
Fortified skimmilk plus hydrogenated cottonseed and soybean oil blend	87.3	9.2	3.5	22.0

[a] TDN content estimated using digestion coefficients for skimmilk and nonfat dried solids as listed in the 22nd edition of Morrison's *Feeds and Feeding*, and assuming a digestion coefficient of 90 for each added fat.

TABLE 3

Weight gains and efficiency of feed utilization among the various groups for a 76-day experimental period

Group	Diet	No. calves	Av. total gain	Av. daily gain	Av. digestible dry matter intake	Av. gain per lb. digestible dry matter
			(lb.)			
I	Skimmilk	3	67.0	0.88	101.5	0.66
II	Corn oil filled milk (before change in infection control)	5[a]	42.0	0.55	113.5	0.37
III	Corn oil filled milk (after change in infection control)	6	61.0	0.80	122.0	0.50
IV	Corn oil filled milk with intramuscular tocopherol supplementation	4	39.5	0.52	101.3	0.39
V	Corn oil filled milk with oral tocopherol supplementation	6	60.0	0.79	115.4	0.52
VI	Hydrogenated corn oil filled milk	4	110.0	1.45	146.7	0.75
VII	Lard filled milk	6	101.0	1.33	148.5	0.68
VIII	Hydrogenated cottonseed and soybean oil blend filled milk	4	126.0	1.66	175.0	0.72
IX	Whole milk	4	126.0	1.66	165.8	0.76
X	Butter oil filled milk	3	152.0	2.00	192.4	0.79
XI	Fortified skimmilk	4	146.0	1.92	239.3	0.61
XII	Fortified skimmilk plus corn oil	4	128.0	1.68	220.7	0.58
XIII	Fortified skimmilk plus hydrogenated cottonseed and soybean oil blend	2	211.0	2.78	293.1	0.72

[a] Only five of the 12 calves in this group completed the experimental period.

Debilitation, infection, and mortality. Considerable differences were noted in the character and amounts of feces voided by calves on the various rations. All calves on the corn oil filled milks, with and without tocopherol supplementation, were afflicted with a marked diarrhea throughout the experimental period. A marked diarrhea persisted in animals fed the fortified skimmilk plus corn oil ration also. The feces voided by calves in Groups I, XI, and XIII varied diurnally, from firm to markedly loose. In contrast, the feces of animals in Groups VI through X were firm throughout the experiment.

Calves in all corn oil filled milk groups were unthrifty and emaciated. Those in the skimmilk group also were somewhat unthrifty. Although the skimmilk animals did not seem emaciated, they did appear stunted. All calves on the remaining diets studied were thrifty and in good flesh. Subjects fed the fortified skimmilk plus hydrogenated vegetable oil ration, however, appeared extremely fat.

Various calves in Groups II, III, and VII developed some or all of the following symptoms: anorexia, general weakness, unsteady gait, standing with arched back, open shoulders, or prominent suprascapula, stiffness in the fore and/or rear quarters, muscle tremors, difficulty in swallowing and inability to stand. These symptoms usually appeared after approximately 55 days of experimental feeding. Fifty per cent of the animals in the corn oil filled milk groups were so afflicted, while only one of six calves on the lard filled milk diet exhibited such symptoms. In addition, one of the three subjects fed butter oil filled milk developed an acute case of muscular involvement. It should be added that the skin of several corn oil filled milk animals seemed leathery, and the underlying muscles appeared "petrified" during the terminal stages of the syndrome. Thus, it was nearly impossible to reach the jugular vein, even with the sharpest of bleeding needles. None of the animals on the other diets was afflicted with any of the symptoms mentioned.

More than 50% of the calves receiving corn oil filled milk before the change in infection control contracted pneumonia or other respiratory infections. This is in sharp contrast to no cases in a similar group following the change in infection control. Single cases occurred also in the lard and corn oil filled milk with intramuscular tocopherol supplementation groups, as well as on the fortified skimmilk plus corn oil ration. None of the subjects on the other diets contracted pneumonia or other respiratory disorders. Rectal temperatures of 103.5° F. or above were frequently encountered in all groups receiving corn oil or lard filled milk. High rectal temperatures were almost nonexistent in all other groups.

Some animals in certain groups died and others were sacrificed when death was expected momentarily, to ensure accurate post-mortem findings. Prior to the change in infection control, 90% of the corn oil filled milk calves died. Most of these losses occurred during the 76-day experimental period. Several deaths, however, occurred following 12-28 days of additional feeding. Following the change in infection control, only two of six animals in a subsequent corn oil filled milk group died or were sacrificed during the experimental period. One of three calves in the butter oil filled milk group was afflicted with acute muscular involvement and was sacrificed after 89 days of experimental feeding.

In some cases, the subjects were maintained on the experimental rations to 5-6 mo. of age, to determine if any deleterious effects might be manifested as the result of a longer feeding period. No adverse effects were noted, however, when certain calves on the hydrogenated corn oil, hydrogenated cottonseed, and soybean oil blend and lard filled milk diets remained on those rations to 5-6 mo. of age. This was also true in the case of the whole milk and fortified skimmilk diets. In addition, two animals in the butter oil filled milk group were carried to 5 and 6 mo., respectively, despite the earlier loss of one calf on this diet.

The observations concerning the degree of diarrhea, general appearance, and flesh of the experimental calves closely agree with those previously reported by others (7, 8, 9, 11, 13). The symptoms noted in the present study are also strikingly similar to those observed in calves afflicted with nutritional muscular dystrophy (2, 14).

The greater incidence of pneumonia in the corn oil filled milk group suggests an increased susceptibility to respiratory infection in calves receiving this diet. Blaxter, Watts, and Wood (2) have suggested that the high incidence of pneumonia in dystrophic calves may be related to food entering the lungs due to poor control of throat and tongue muscles. A portion of the feed refusals noted in the terminal stages of the syndrome in the present study also might be attributed to an involvement of throat and tongue muscles. The effectiveness of the infection control program used with a later group of corn oil filled milk animals, as well as with all other groups, is indicated by the relatively few cases of pneumonia which occurred following its adoption. The marked reduction in mortality, which resulted after the change in infection control, strongly suggests that infection may have been responsible for a large number of the deaths in the initial corn oil filled milk group.

The finding that calves receiving several of the diets were able to remain on the experimental rations to 5-6 mo. of age without adverse effects is not entirely in agreement with a report by Thomas and Okamoto (15). These workers have found that animals fed whole milk exclusively died when 5 mo. to 1 yr. of age. It should be pointed out that while only one death occurred in three calves fed butter oil filled milk, one of the remaining subjects exhibited muscular lesions upon necropsy. The possible cause for the unsatisfactory results obtained with the butter oil filled milk will be discussed further, in later reports.

Weight gains and feed utilization. The presentation of these data has been complicated by the differences in dry matter digestibility and intake, as well as other factors. Certain diets were extremely low or high in total solids or estimated total digestible nutrient content for experimental reasons. A summary of the weight gain and feed utilization data may be found in Table 3.

The gains made by calves fed the various corn oil filled milks were considerably less than those of Groups VI through X, inclusively. The average gain for the skimmilk fed animals was somewhat greater than that for the groups fed corn oil filled milk, despite a lower digestible dry matter intake by the subjects receiving skimmilk. Gains made by calves in Group XII did not exceed those made by subjects in Group XI, and were substantially less than the gains of Group

XIII. These findings suggest that the feeding of a corn oil filled milk to dairy calves results in a depression of growth and/or poor utilization of dietary fat. The poor gains made by the corn oil filled milk animals are in general agreement with results obtained in earlier experiments using corn, cottonseed, and soybean oils (7, 8, 9, 11, 13).

It is of considerable interest to note that satisfactory gains as well as physical condition were maintained in calves fed the fortified skimmilk ration to 5-6 mo. of age. This diet contained only 0.15% fat. Also, no symptoms of a fat deficiency were observed in calves raised to 90 days of age on the skimmilk diet containing 0.10% fat. These results suggest that a fat content of 0.10–0.15% in an otherwise adequate ration may be sufficient to prevent the development of a fat deficiency in calves as described by other workers (4, 10). It should be emphasized, however, that all animals in this investigation received colostrum and were fed whole milk to approximately 14 days of age. Evidence similar to that obtained with the low-fat rations in this study has recently been reported by Wing, Jacobson, and Allen (16).

An analysis of variance of the feed utilization data revealed that significant differences existed among diets. Further analyses were conducted according to procedures outlined by Duncan (5, 6), to test the differences among various treatment means at the 5% level of significance only. The average gains per pound of digestible dry matter made by the butter oil filled milk and whole milk groups were significantly greater than those of animals fed corn oil filled milks or the fortified skimmilk with corn oil ration. Significant differences in feed utilization were also found among the groups fed hydrogenated vegetable oil filled milks and those receiving corn oil filled milks. In addition, feed utilization by skimmilk and lard filled milk calves was significantly greater than that found on all corn oil filled milk diets, except the corn oil filled milk group receiving oral tocopherol supplementation. It was also established that no significant differences in feed utilization existed among Groups I, VI-XI (inclusive), and XIII.

Apparently the change in infection control beneficially affected gains made by corn oil filled milk calves. It did not, however, significantly improve the efficiency of feed utilization. Tocopherol supplementation of the corn oil filled milk diet also did not improve weight gains or the efficiency of feed utilization, in spite of the absence of muscular involvement. This finding tends to substantiate recent reports (3, 14), in which weight gains of calves afflicted with nutritional muscular dystrophy were not adversely affected. The intramuscular administration of tocopherol resulted in markedly reduced weight gains. This reduction in gain undoubtedly is associated with the mode of supplementation rather than the tocopherol per se. Calves in this group had a greater incidence of high rectal temperatures than the other corn oil filled milk groups.

The growth rate of animals on the fortified skimmilk plus hydrogenated cottonseed and soybean oil blend diet was indeed remarkable. The average daily gain made by the subjects in this group was 2.78 lb., as compared to 2.00 and 1.92 lb. for calves fed the butter oil filled milk and fortified skimmilk

rations, respectively. The weight gains made by the animals in the fortified skimmilk groups suggest that a detailed study of similar diets might of a value from the standpoint of producing choice veal.

Supplementation studies. In addition to the vitamin and mineral supplements already mentioned, several other supplements were fed to additional calves, to determine the extent to which they might alleviate the deleterious effects noted in animals receiving corn oil filled milk. Four calves on a corn oil filled milk ration were fed alfalfa hay throughout the experimental period. These animals were on experiment before the change in infection control was initiated. None of the subjects in this group, however, was afflicted with pneumonia or other respiratory disorders. The feces of these calves were normal in color and varied from loose to reasonably firm in consistency. Two of the animals completed the experimental period of 76 days, and were subsequently sold.

After approximately 66 days of experimental feeding, the physical condition of the remaining pair of calves deteriorated to the extent that it was necessary to remove them from the diet. The average daily gain of the entire hay-supplemented group was 0.74 lb., as compared to 0.55 lb. for comparable subjects fed corn oil filled milk alone. However, the average daily gain for the calves completing the experimental period was 0.85 lb. Two calves which completed the feeding trial consumed an average of 1.9 lb. of hay daily, as compared to 0.5 lb. for those unable to complete the experimental period. Apparently, the consumption of appreciable quantities of alfalfa hay partially alleviated the deleterious effects of corn oil feeding in this study. This indication is not entirely in agreement with the results obtained by others (1), who found that the deleterious effects of feeding a highly unsaturated vegetable oil could not be overcome by the inclusion of alfalfa hay and grain in a ration for dairy calves.

Sodium acetate was administered orally to four calves which were in poor physical condition as the result of corn oil filled milk feeding. In all instances, 21.3 g. of sodium acetate were dissolved in each of the morning and evening allowances of the diet. An immediate improvement in appetite was noted in two of the animals. In addition, a slight increase in daily weight grains occurred in three of the four subjects. These improvements, however, were transitory in all calves, as their condition again deteriorated following approximately 30 days of sodium acetate supplementation.

An additional group of corn oil filled milk calves was supplemented with soya phosphatides, added to the filled milk prior to homogenization at a rate to provide a liquid product containing 0.1% soya phosphatides. This level gave the animals a minimal intake of approximately 5 g. of this supplement per head daily. No noticeable improvement in the condition of the subjects resulted from soya phosphatide supplementation throughout the experimental period.

Streptomycin was administered orally to an additional group of animals on the corn oil filled milk ration. A solution of streptomycin in sterile distilled water was added to the filled milk immediately before feeding, at a rate to provide 45 mg. of streptomycin per pound of milk fed. Such supplementation did not reduce the severity of the diarrhea or have any other beneficial effects upon

the well-being of the calves. Similarly, no improvement in physical condition or weight gains was observed in two corn oil filled milk animals supplemented with high levels of ascorbic acid, in an attempt to counteract the possible toxic effects of corn oil feeding. The ascorbic acid was administered orally, so as to provide 3.6 g. per kilogram of fat fed throughout the experimental period.

It should be pointed out that nine additional calves on the corn oil filled milk diet were removed from the experimental ration at a time when they were exhibiting the typical symptoms already mentioned. A ration consisting of whole milk, alfalfa hay, and a grain mixture was then introduced. The improvement in the condition of six of the animals was almost immediate and very striking. Three of the subjects, however, showed no response to this change in diet. Most calves regained their strength within 5–11 days, and the color and consistency of the feces usually returned to normal within 1–2 wk. The daily weight gains made by the animals doubled or, in some instances, tripled within 2 wk. following the change in regime. Complete recovery usually resulted in 20–40 days. The recovery of corn oil filled milk animals following a change to a ration of whole milk, hay, and concentrates appeared to be dependent upon the extent of muscular involvement and the presence or absence of pneumonia.

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SOME EFFECTS OF FEEDING VARIOUS FILLED MILKS TO DAIRY CALVES. II. FECAL CHARACTERISTICS AND DIGESTIBILITY DATA[1]

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SUMMARY

Young dairy calves were fed low-fat rations or filled milks containing various fats. One group received a corn oil filled milk which was prepared daily rather than weekly, to study effects of storage. All other diets were prepared once each week. Differences in the color of feces were observed among the various rations. Abnormally large amounts of feces were voided by animals fed the corn oil filled milk prepared weekly. Daily preparation of the corn oil filled milk diet or the feeding of hydrogenated corn oil greatly reduced the amounts of feces excreted.

Apparent dry matter digestibility for the weekly prepared corn oil filled milk group was significantly less than that obtained for any of the other diets. The apparent metabolic fecal fat excretion by calves fed a skimmilk diet containing 0.1% butterfat amounted to 5.36% of the fecal dry matter. It was established that the apparent digestion coefficients for butter oil were significantly greater than those for corn oil, hydrogenated corn oil, or lard. Daily preparation of the corn oil filled milk significantly increased dry matter digestibility, but did not significantly increase fat digestibility over the weekly preparation of this ration. Partition of the fecal fat revealed that a considerable portion was excreted as soaps by animals receiving the various diets. The fecal fat partition of calves fed butter oil filled milk was similar to that of apparent metabolic fecal fat, as excreted by animals in the skimmilk group.

The effects of various filled milks upon the physical condition, appearance, and growth of calves have been presented in an earlier paper (1). The research reported here represents a further attempt to ascertain some of the factors responsible for the unsatisfactory weight gains and poor feed utilization encountered when highly unsaturated vegetable oils are fed to dairy calves.

EXPERIMENTAL PROCEDURE

A number of calves from several of the groups mentioned in an earlier paper were used in the digestibility studies reported here. Details on the feeding and management of the subjects were presented in a previous report (1).

Since the animals from several of the groups were highly susceptible to respiratory disorders and often in a weakened condition, the customary collection period used in digestion trials was not employed. Instead, the calves were periodically placed in metabolism stalls for a three-day period at approximately 20-day intervals, beginning at 35 days of age. Total collection of the feces voided by the subjects was made during the 48 hr. following a preliminary

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period of 24 hr. Feed intake was held constant for approximately 1 wk. prior to the collection period. This manner of collection did not unduly jeopardize the health of the individuals and prevented interference with other phases of the experiment, particularly those dealing with the development of muscular involvement. It also afforded an opportunity to determine whether digestibility was progressively affected during the experimental period. Following the weighing of the feces voided during the collection period, representative samples were taken and stored at −20° C. until analyses were performed. The storage period for the fecal samples did not exceed 8 wk.

Dry matter was determined by oven drying. A sample of the dried feces was pulverized and used in the determination of total fat content and the various fat components. A slight modification in the method of Van der Kammer and associates (12) made it possible to ascertain the grams of soaps as well as free fatty acids and neutral fat present per 100 g. of fecal dry matter. The original method gives values for neutral fat and the total fatty acids present as fecal soaps and free fatty acids. In the modification an additional titration was performed on a portion of the dried fecal sample, which was refluxed without the addition of HCl to avoid the liberation of the fatty acids present as soaps. The value obtained from this additional titration was taken as the original free fatty acids present in the fecal sample. The difference between this value and the one obtained after refluxing with acid was taken as the original soaps present in the sample of dried feces.

RESULTS AND DISCUSSION

Fecal characteristics. As mentioned in the earlier report (1), marked differences in the consistency of the feces were found among the various dietary regimes. The mean daily fecal excretion values in Table 1 provide a reasonable

TABLE 1
Fecal output by calves on the various diets

Group	Diet	No. calves	No. trials	Mean dry matter intake	Mean daily fecal excretion [a]	Standard error of mean
				(g.)	*(g.)*	
I	Corn oil filled milk (prepared weekly)	10	41	829.8	1,176.3	± 22.8
II	Corn oil filled milk (prepared daily)	4	16	839.1	477.2	± 15.1
III	Hydrogenated corn oil filled milk	3	12	1,161.2	698.3	± 23.9
IV	Lard filled milk	4	10	1,256.6	557.6	± 20.6
V	Butter oil filled milk	3	12	896.8	469.2	± 20.6
VI	Fortified skimmilk	2	6	2,031.8	1,772.8	±319.7
VII	Fortified skimmilk plus corn oil	5	22	1,689.1	1,398.1	± 69.0
VIII	Fortified skimmilk plus hydrogenated soybean and cottonseed oil blend	2	8	2,063.5	1,428.4	±162.3
IX	Skimmilk	2	11	667.0	907.8	± 55.9

[a] Wet basis.

indication of the amount of diarrhea present among the various groups. The fecal output of subjects in groups with feces of apparently normal consistency (Groups II–V, inclusive) was somewhat greater than that reported by Blaxter and Wood (4) in their study of diarrhea in calves. Animals fed corn oil filled milk prepared weekly were afflicted with a severe diarrhea. In some trials, as much as 3,000 g. of feces were collected daily from individuals in this group. Tocopherol supplementation of the corn oil filled milk diets did not reduce the severity of the diarrhea. Calves receiving a corn oil filled milk prepared daily in an attempt to reduce its toxicity voided a considerably smaller amount of feces. Hydrogenation of the corn oil greatly reduced the amount of feces excreted daily. Similarly to Group I, the feces of animals on the fortified skimmilk plus corn oil diet were extremely loose.

Although the mean daily excretion of feces was greatest for the fortified skimmilk group, there was a large amount of variation in the values for that group, as shown by the standard error of the mean. Considerable variation in the consistency of the feces was also noted in the trials of animals in Group IX. The marked fecal excretion by calves fed the various fortified skimmilk diets undoubtedly was influenced to some extent by the abnormally high solids content of these rations. The factors responsible for the relatively large fecal output by the skimmilk group are not apparent. It is interesting to note, however, that Iowa workers (13) recently reported that diets high in concentrations of milk solids-not-fat and/or low in fat are likely to cause diarrhea.

Differences in the color of feces were also quite pronounced among the various experimental rations. The feces of animals on the weekly prepared corn oil filled milk diet were characteristically greenish-yellow in color; whereas, those excreted by calves receiving the same diet prepared daily were a darker green. The excretions of the lard filled milk group were usually grayish-blue or sometimes clay-like. Subjects on the whole milk, skimmilk, fortified skimmilk, and butter oil filled milk rations voided feces which were the usual brown in color. In contrast, the fecal excretions of individuals on the hydrogenated vegetable oil filled milks were invariably gray. Differences in bile secretion, intestinal fat excretion, intestinal flora, and the oxidation-reduction potential of the digestive tract, as well as other factors, may influence color of feces.

Digestibility data. The apparent dry matter digestion coefficients for several of the various diets may be found in Table 2. These data were treated statistically

TABLE 2

Apparent dry matter digestion coefficients of the various experimental diets

Diet	No. calves	No. trials	Av. dry matter digestion coefficient
Corn oil filled milk prepared weekly	8	41	81.1
Corn oil filled milk prepared daily	4	16	90.4
Hydrogenated corn oil filled milk	3	12	89.5
Lard filled milk	4	10	90.9
Butter oil filled milk	3	12	93.6

according to methods outlined by Snedecor (11) and Duncan (9, 10), to test the differences among the various treatment means at the 5% level of significance. The average dry matter digestion coefficient for the weekly prepared corn oil filled milk group was found to be significantly less than that for any one of the other diets. No other significant differences in apparent dry matter digestibility were noted among the various diets. The dry matter digestion coefficients for calves in the various fortified skimmilk groups were not included in the analyses because of the limited amount of data available for these diets.

The significantly greater apparent dry matter digestibility of the daily prepared corn oil filled milk ration is of considerable interest. Undoubtedly, a large portion of the increase in apparent dry matter digestibility may be associated with the decrease in fecal excretion and degree of diarrhea. As previously noted, the feces of calves in the daily prepared corn oil filled milk group were nearly normal in consistency. Blaxter and Wood (4) reported that the apparent digestibility of fat, protein, and ash drops from as high as 97 to as low as 40% in some instances, as the result of diarrhea in calves.

The apparent fat digestibility data are presented in Table 3. The adjusted fat digestion coefficients listed in this table are values adjusted for metabolic fecal fat as follows: The mean amount of fat excreted daily by calves in the skimmilk group was expressed as a percentage of the fecal dry matter. Since the diet of these animals contained only 0.1% fat, it was assumed that this value, 5.36%, was indicative of the metabolic fecal fat expressed as a percentage of the fecal dry matter. Thus, an amount equivalent to 5.36% of the fecal dry matter was deducted from the total fat excretion in calculating the adjusted fat digestion coefficient for calves receiving the several filled milks.

The data used in determining the metabolic fecal fat excretion were derived from 11 digestion trials conducted with two subjects in the skimmilk group. The finding that the metabolic fecal fat excretion by calves in this group amounted to 5.36% of the fecal dry matter is in very close agreement with the 5.30% value obtained under similar conditions by previous workers (5). More recently, Cunningham and Loosli (7) reported values of 3.1–5.8% when the metabolic fecal fat excreted by calves on a fat-free purified diet was expressed as a percentage of the fecal dry matter.

An analysis of variance of the unadjusted fat digestibility data indicated that significant differences existed among the various diets. Further statistical treatment revealed that the digestibility of butter oil was superior to that of the fats in each of the other diets studied. In every case this superiority of butter oil was significant at the 1% level. No other significant differences in unadjusted fat digestion coefficients existed among the remaining diets.

Significant differences were found to exist also among the adjusted fat digestion coefficients for the various rations. The digestibility of butter oil was again demonstrated to be significantly greater (1% confidence level) than that of the fats in the other diets. No other significant differences were noted among the remaining groups. It should be pointed out that both the highest and lowest fat digestion coefficients for the over-all experiment occurred in the weekly

TABLE 3

Summary of apparent fat digestibility data for calves on the various experimental diets

Diet	No. calves	No. trials	Av. unadjusted fat digestion coefficient	Av. adjusted [a] fat digestion coefficient	Free fatty acids	Soaps	Neutral fat
					(av. % fecal fat)		
Corn oil filled milk prepared weekly	8	32	84.3	88.3 ± 0.30[b]	22.9 ± 0.53[b]	42.8 ± 0.85[b]	34.3 ± 0.48[b]
Corn oil filled milk prepared daily	4	14	88.5	90.2 ± 0.36	12.7 ± 0.87	57.7 ± 1.20	29.6 ± 0.73
Hydrogenated corn oil filled milk	3	10	88.2	90.3 ± 0.54	25.7 ± 2.26	53.7 ± 2.22	20.6 ± 1.23
Lard filled milk	4	7	87.1	88.9 ± 1.06	20.2 ± 2.85	52.6 ± 2.99	27.2 ± 0.79
Butter oil filled milk	3	9	95.1	96.8 ± 0.26	25.3 ± 2.62	34.3 ± 1.70	40.4 ± 1.69
Skimmilk	2	8	—	—	24.2 ± 3.23	26.6 ± 2.92	49.2 ± 2.57

[a] Values adjusted for metabolic fecal fat as noted in text.

[b] Mean ± standard error of the mean.

prepared corn oil filled milk group. No consistent effect of collection period upon fat or dry matter digestibility was observed in any of the groups.

It is interesting to note that the apparent digestibility of the hydrogenated corn oil did not differ significantly from that of corn oil or lard. The melting point range of the hydrogenated corn oil used in this investigation was 45–48° C. Studies reported by Crockett and Deuel (6), as well as by others, have indicated that the critical melting point range above which there appears to be a marked decrease in fat digestibility is 50–55° C. The apparent digestion coefficients for hydrogenated corn oil obtained in this experiment compare favorably with those obtained by Augur, Rollman, and Deuel (2), when rats were fed hydrogenated cottonseed oil with a similar melting point. They are, however, somewhat greater than those for hydrogenated soybean oil as reported for calves by de Man (8).

The digestibility of lard in this study is somewhat less than that reported by Crockett and Deuel (6) for the rat, but somewhat greater than the 82.8% figure obtained by Cunningham and Loosli (7), when calves were fed a purified diet. The adjusted digestion coefficient of 96.8 for butterfat is somewhat greater than the 93.0 figure for calves obtained similarly by earlier workers (5). However, both the adjusted and unadjusted digestion coefficients for butterfat are in good agreement with values reported by de Man (8) as well as by Blaxter and Wood (3). It should be pointed out that the daily preparation of the corn oil filled milk did not significantly increase the digestibility of this highly unsaturated vegetable oil, although dry matter digestibility was significantly improved, as noted earlier.

The partition of fecal fats is also presented in Table 3. The finding that a considerable portion of the fecal fat is present as soaps is in agreement with previous results obtained with rats (2, 6) and calves (3). Thus, it appears important that fat determinations be made on acidified samples of fecal dry matter in fat digestibility studies, in order to include fecal soaps in the total fat excretion. It should be noted that a larger portion of fecal fat was excreted as soaps than as neutral fats and free fatty acids combined, by animals on the hydrogenated corn oil diet. Augur, Rollman, and Deuel (2) also showed this to be true in digestibility studies of hydrogenated cottonseed oil with rats. This same pattern of fecal fat excretion occurred in the daily prepared corn oil and lard filled milk groups, and was closely approximated by animals on the weekly prepared corn oil filled milk ration. Blaxter and Wood (4) obtained similar results with calves fed margarine.

A marked decrease in the percentage of fecal fat excreted as free fatty acids occurred when the corn oil filled milk diet was prepared daily. Undoubtedly, a portion of this decrease is associated with the abatement of the severe diarrhea in this group. Blaxter and Wood (4) demonstrated that a large increase in fatty acid excretion results in calves afflicted with diarrhea. Regarding the partition of the metabolic fecal fat as listed under the skimmilk diet in Table 3, the percentage of fecal fat excreted as free fatty acids closely agrees with the value obtained by Cannon, Espe, and Waide (5). The percentage of metabolic fecal fat present as neutral fat, however, is approximately 10% greater than in

the results reported by these workers. On the other hand, the portion of metabolic fecal fat present as neutral fat in this investigation is in agreement with the results obtained by Cunningham and Loosli (7). It is interesting to note the similarity between the fecal fat partition on the butter oil filled milk diet and that of what has been assumed to be metabolic fecal fat, as excreted by subjects in the skimmilk group.

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SOME EFFECTS OF FEEDING VARIOUS FILLED MILKS TO DAIRY CALVES. III. BLOOD PLASMA TOCOPHEROL AND VITAMIN A LEVELS, DIET STORAGE EFFECTS, AND EVIDENCE OF TOXICITY [1]

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SUMMARY

Low blood plasma tocopherol levels were found in calves fed corn oil filled milk, despite a relatively high tocopherol intake. Both plasma tocopherol and vitamin A values declined progressively during the experimental period in the corn oil filled milk group. Low blood plasma tocopherol levels also occurred in animals fed lard filled milk and a ration containing butter oil, which was prepared from oxidized butter. Oral supplementation of the corn oil and lard filled milk diets with 500 mg. of DL-alpha tocopherol or alpha tocopheryl acetate daily resulted in high plasma tocopherol values.

A loss of 30-40% of total tocopherol content of the corn oil filled milk resulted during a maximum storage period of seven days. It was also demonstrated that an extremely high peroxide content developed in the corn oil filled milk during a similar storage period. The feeding of a corn oil filled milk prepared daily rather than at weekly intervals markedly reduced the severity of the diarrhea and increased dry matter digestibility. Weight gains, however, were not materially affected.

The weekly prepared corn oil filled milk was found to be as detrimental for calves 4 mo. old as for younger animals. The possibility that corn oil filled milk is toxic to dairy calves is discussed in considerable detail.

Since the presence of highly unsaturated fats and rancid fats, in general, has been associated by various workers (18, 19) with the destruction of fat-soluble vitamins or alterations in the body requirements for such factors, a study was undertaken of blood plasma tocopherol and vitamin A levels in calves fed various filled milks. The symptoms of muscular involvement reported previously (1) as occurring in animals fed corn oil filled milk further suggested the necessity of such a study.

Other phases of the investigation presented here were conducted because of evidence reported by other workers (7, 21) that highly oxidized fats also exert a direct toxic effect upon the body.

EXPERIMENTAL PROCEDURE

The methods used in the feeding and management of the subjects have been published earlier (1, 2). Approximately 50-ml. samples of jugular blood were

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drawn at intervals of from ten to 20 days throughout the experimental period. All pre-experimental samples were obtained when the calves were from ten to 14 days of age. The plasma was separated from the cellular components and stored at −4° C. until assayed for tocopherol and, in some cases, vitamin A content. The storage period never exceeded 2 wk.

For the determination of blood plasma tocopherol levels, aliquots containing 5.0 ml. of blood plasma were extracted with petroleum ether (b.p. 63–67° C.), as outlined by Kimble (16) and Quaife and Harris (24). The total fat-soluble reducing substances were determined colorimetrically, using the Emmerie and Engel reaction (11), in accordance with procedures employed by Devlin and Matill (9). Based on research reported by Kaunitz and Beaver (15), a correction was made for color inhibition by plasma extracts. Since most calves had low blood plasma carotene values, the correction for carotene interference was small, or negligible. All values were expressed as micrograms of alpha tocopherol per 100 ml. of blood plasma. In addition to the blood plasma tocopherol estimates, determinations of total fat-soluble reducing materials were also made on aliquots of the corn oil filled milk, employing generally the same procedures used in blood plasma analyses.

Blood plasma vitamin A determinations were made on 10-ml. samples obtained as outlined above. The method used was essentially that described by Kimble (16), but with mild saponification as given by Bessey *et al.* (5). The vitamin A was measured colorimetrically by means of the Carr-Price reaction. The total Carr-Price reacting materials were calculated and expressed as micrograms of vitamin A per 100 ml. of blood plasma.

Several methods of tocopherol administration and types of tocopherol supplements were investigated. In every instance, an equivalent of 500 mg. of DL-alpha tocopherol or alpha tocopheryl acetate was administered daily to each calf. All orally administered tocopherol supplements were fed in gelatin capsules immediately prior to the P.M. feeding. Sesame oil served as a carrier for both the oral and intramuscular administration of alpha tocopheryl acetate. No carrier was used with the orally administered DL-alpha tocopherol. Whereas the orally supplemented calves were given 500 mg. of tocopherol supplement daily, the subjects in the intramuscularly administered group received 1,500 mg. of alpha tocopheryl acetate every three days.

Peroxide values were obtained on samples of the filled milks by a modification of the Hills and Thiel method (12), as developed by Stine *et al.* (26). This method is based on the oxidation of the ferrous ion to ferric in the presence of peroxides, and the resulting formation of ferric thiocyanate.

RESULTS AND DISCUSSION

Tocopherol determinations. The mean plasma tocopherol levels of the various groups for the experimental period of 76 days, and the "t" values for certain comparisons, are given in Table 1. The relatively low mean tocopherol estimate for the corn oil filled milk group is of considerable interest, in view of the tocopherol content of corn oil (29). The mean for the corn oil filled milk group

TABLE 1

Mean total fat-soluble reducing substances, expressed as micrograms of alpha tocopherol per 100 ml. blood plasma

Diet	No. calves	No. determinations	Mean tocopherol value micrograms %	t-value
I. Comparison of corn oil filled milk with various other diets				
Corn oil filled milk	9	34	137	.....
Hydrogenated corn oil filled milk	4	19	187	2.09*
Hydrogenated cottonseed and soybean oil blend filled milk	3	6	468	3.21**
Lard filled milk	4	20	93	3.34**
Butter oil filled milk	3	15	90	4.35**
Fortified skimmilk	3	14	45	7.76**
Whole milk	2	14	314	5.97**
II. Comparison of the oral tocopherol supplemented corn oil filled milk ration with various other diets				
Corn oil filled milk with oral DL-alpha tocopherol supplementation [a]	3	14	774	.....
Corn oil filled milk with oral alpha tocopheryl acetate supplementation [a]	4	15	708	0.78
Corn oil filled milk with intramuscular alpha tocopheryl acetate supplementation [a]	3	15	379	5.48**
III. Comparison of lard filled milk ration with the oral tocopherol supplemented lard filled milk diet				
Lard filled milk	4	20	93	.....
Lard filled milk with oral alpha tocopheryl acetate supplementation [a]	2	10	584	9.44**

* Significant at the 5% level.

** Significant at the 1% level.

[a] All animals received an equivalent of 500 mg. of alpha tocopherol daily. See Procedure section for further details.

given in Table 1 does not include determinations obtained from the last three subjects raised on this diet. A mean estimate of 200 μg. of alpha tocopherol per 100 ml. of blood plasma resulted in these particular calves. It is felt that this discrepancy in results might stem from a change in the time of blood collection. Whereas all other blood samples were collected approximately 3 hr. after the morning feeding, these were obtained approximately 6 hr. following the morning allowance of the ration. Barker and Jacobson (3) have demonstrated that the plasma "fat" values of calves receiving a highly unsaturated vegetable oil increase to a maximum at the end of 6 hr. after feeding. Because of the tocopherol content of corn oil and this plasma fat pattern, it is quite conceivable that the time of collection may have influenced the plasma tocopherol levels of these subjects.

The considerable difference noted between the mean plasma tocopherol values obtained for the hydrogenated corn oil and hydrogenated cottonseed and soybean oil blend calves can not be explained on the basis of available data. It should be noted, however, that the levels found in the latter group are of the order expected, in view of the tocopherol content of vegetable oils. The hydrogenated corn oil used in this study was prepared from refined corn oil, specifically for the purpose of this investigation. The tocopherol content of the corn oil may have been deleteriously affected by the processes used in the hydrogenation of the product in the pilot-sized operation. No data, however, are available on the tocopherol content either of the hydrogenated corn oil or of the hydrogenated cottonseed and soybean oil blend. The difference in mean plasma tocopherol values between the whole milk and butter oil filled milk groups also requires elaboration. Undoubtedly, the low plasma tocopherol levels found in butter oil filled milk calves may have resulted from a possible low tocopherol content of the butter oil. This product was prepared from oxidized butter and gave higher than usual peroxide values when incorporated in the filled milk. The low blood plasma tocopherol values found in the lard filled milk group presumably were largely a reflection of the notoriously low tocopherol content of lard. Similarly, the extremely low levels obtained from the calves raised on the fortified skimmilk ration were clearly a reflection of the low tocopherol content of the diet.

When administered orally, DL-alpha tocopherol and alpha tocopheryl acetate were equally effective in maintaining high levels of tocopherol in the blood plasma of animals fed corn oil filled milk. However, intramuscular administration of alpha tocopheryl acetate failed to raise the plasma tocopherol values to levels as high as did oral supplementation. British workers (6) reported that no increase in blood plasma concentrations of tocopherol occurred when alpha tocopheryl acetate was administered intramuscularly. Other investigators (25) found that subcutaneous injections of alpha tocopheryl acetate gave only slight or no increase in blood plasma tocopherol values. These discrepancies in the results of parenteral administration may be due, in part, to differences in dosages and sampling times, as well as to other factors.

The tocopherol values for all groups declined during the first 20 days of experimental feeding. This period coincided with the 15-35 days of age interval

for the subjects. Parrish *et al.* (22) have demonstrated that such a decrease in fat-soluble vitamins occurs in the blood plasma of normal calves during the 1st mo. of life. A statistically significant difference in blood plasma tocopherol levels due to sampling periods beyond 35 days of age was found only in the corn oil filled milk group. A mean of 180 μg. of tocopherol per 100 ml. of blood plasma following 20 days of corn oil feeding diminished to a mean of 115 μg. at the end of 70 days. This steady decline in plasma tocopherol values occurred despite the ingestion of increasingly greater amounts of a vegetable oil with a relatively high tocopherol content. A similar decrease in plasma tocopherol concentrations was encountered by Viswanatha, Gander, and Liener (28) when rats were fed a corn oil–lactose diet.

To ascertain the effects of storage on the tocopherol content of the weekly prepared corn oil filled milk, serial determinations of the total fat-soluble reducing substances were made on the ration stored at 5° C. As shown in Table 2, the results indicate that a 30–40% decrease in total tocopherol content occurred during a maximum storage period of seven days. The total tocopherol content of the corn oil immediately following the preparation of the diet closely resembled that reported by Willy (29). It has been shown previously, however, that the alpha tocopherol content of corn oil is only 12.6 mg. per 100 g. of oil. The major portion of the total tocopherols in corn oil is of the gamma form. Embree (10), among others, has demonstrated that alpha tocopherol has a higher biological activity than other forms.

TABLE 2

Effect of storage on the tocopherol content of corn oil filled milk

Days stored	Total tocopherol[a]		Tocopherol loss	
	(mg/100 ml. corn oil)		*(%)*	
	Sample 1	Sample 2	Sample 1	Sample 2
0	91	112	0	0
1	79	104	12	8
2	74	88	18	21
3	74	89	18	20
4	71	69	21	38
5	68	....	24	....
7	62	65	31	42

[a] Total fat-soluble reducing materials calculated as gamma tocopherol from 20-min. readings.

Although a 30–40% loss in the total tocopherol content of corn oil may have occurred during the maximum storage period, the alpha tocopherol content of the corn oil fed the subjects should have exceeded that of butterfat, unless it was preferentially destroyed. Lundborg (17) found that butterfat contained 3.0 mg. % of tocopherol, or less, all of which is presumed to be of the alpha form. Thus, the relatively low mean blood plasma tocopherol values obtained in the corn oil filled milk group occurred despite an apparently adequate intake of alpha tocopherol. The decline of plasma tocopherol values may have resulted from a continual increase in tissue requirements for vitamin E, as influenced in some manner by the highly unsaturated fat in the ration. Mason and Filer (18) have suggested that lard and cod liver oil in the diet influence the develop-

ment of vitamin E deficiency symptoms by causing autoxidation of the vitamin during storage, or digestion of the diet and/or increasing the tissue requirements for tocopherol in some unknown manner. However, the possibility that a portion of the decline may have resulted from the prolonged diarrhea must not be overlooked, in view of the report by Thomas and Okamoto (27).

Vitamin A values. As with the tocopherol determinations, good agreement was obtained between duplicate analyses, as well as among values for plasma samples frozen and assayed at regular intervals. For reasons detailed later, plasma vitamin A determinations were conducted only on calves in the corn oil or hydrogenated cottonseed oil filled milk and whole milk groups. Statistical treatment of the vitamin A data revealed that a significant difference due to sampling periods occurred only on the corn oil filled milk diet. This decline in blood plasma vitamin A values is shown in Figure 1. The 15-day values represent the pre-experimental determinations. Similarly to the tocopherol levels, the vitamin A estimates for all groups decreased during the 15–35 days of age period. As noted earlier, results presented by previous workers (22) have indicated that such a decline occurs in normal calves during this period. The decrease in plasma vitamin A concentrations in the corn oil filled milk group follows the same pattern as did the tocopherol values and may be the result of similar factors already mentioned. Statistical treatment of the data indicated that the mean plasma vitamin A level for the corn oil filled milk calves was significantly lower than that of the hydrogenated cottonseed oil or whole milk group at the 5 and 1% confidence levels, respectively.

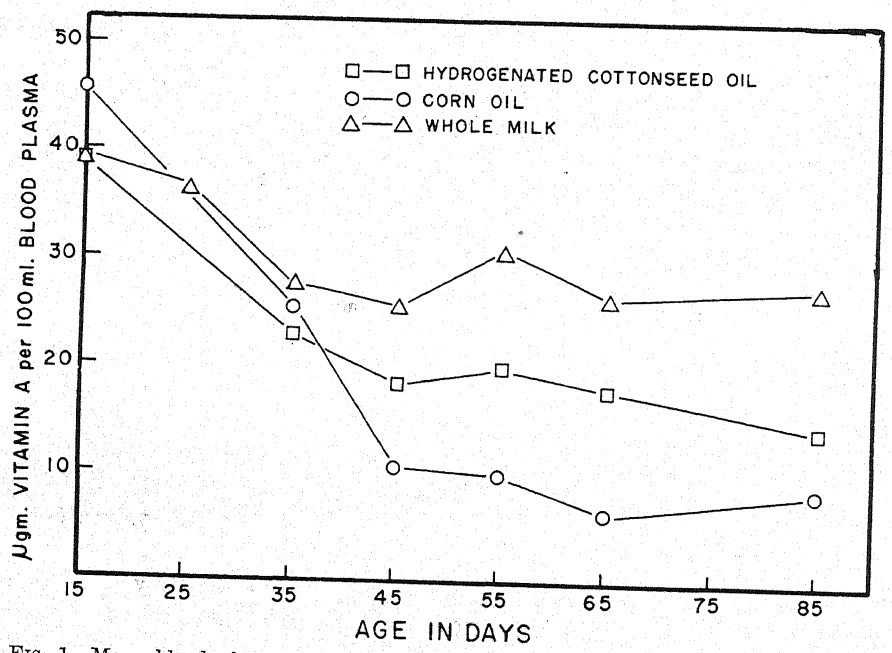


FIG. 1. Mean blood plasma vitamin A estimates of calves fed corn oil or hydrogenated cottonseed oil filled milks and whole milk.

The lowest blood plasma vitamin A values obtained from calves fed corn oil filled milk merely approached the borderline levels demonstrated by Boyer *et al.* (7) to be necessary for adequate vitamin A nutrition. Since a vitamin A deficiency in the corn oil filled milk animals did not appear to be a primary factor on the basis of the blood plasma levels or histopathological findings, further vitamin A determinations were not conducted on subsequent groups. The effects of the corn oil filled milk diet on blood plasma vitamin A concentrations as reported here are similar to those observed by Murley *et al.* (21) in animals receiving soybean oil filled milk. These workers also concluded that a vitamin A deficiency was not a primary factor responsible for the unsatisfactory results obtained when calves were fed a filled milk containing a highly unsaturated vegetable oil. In the present experiment, calves on the filled milks received 160 I.U. of supplemental vitamin A per pound of body weight daily. Whole milk calves received no supplemental vitamin A.

Peroxide content of the several rations. In light of the adverse effect of storage on the tocopherol content of corn oil filled milk, it was deemed advisable to obtain a measure of the peroxide content of the various filled milks. The results of this study are shown in Figure 2. A rapid rise in peroxide values, expressed as milliequivalents of O_2 per kilogram of fat, was noted in the corn oil filled milk during a maximum storage period of seven days at 5° C. The continual increase in the peroxide levels of the corn oil filled milk is in sharp contrast to the relatively constant values obtained with the other filled milks. It should be noted that the peroxide concentrations in the butter oil filled milk were considerably higher than those generally found in butterfat.

Evidence of toxicity. The development of extremely high peroxide values in the corn oil filled milk during storage prompted a study with a corn oil filled

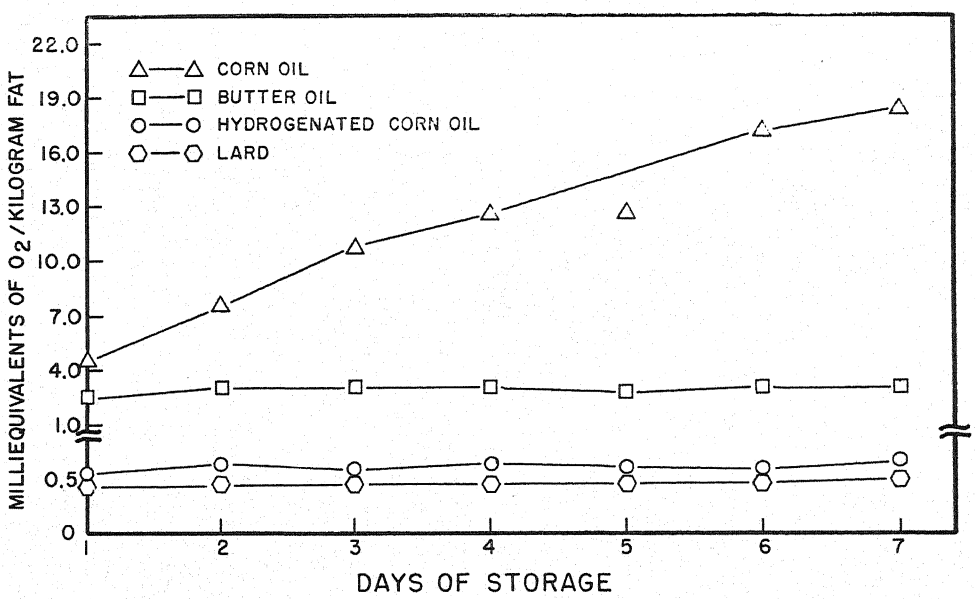


FIG. 2. Peroxide values in various filled milks during a seven-day storage period.

milk prepared daily rather than at weekly intervals. A group of six calves were fed this diet, having a peroxide content which should not have exceeded 5 meq. of O_2 per kilogram of fat. As mentioned in an earlier report (2), the severe diarrhea present in the animals disappeared when the ration was prepared daily. The mean daily fecal excretion of the subjects approached that for individuals receiving butter oil, lard, or hydrogenated corn oil filled milks (2). In addition, the apparent dry matter digestibility of the daily prepared ration was significantly greater than that of the same diet prepared weekly. The apparent fat digestion coefficients for the daily prepared group were also somewhat higher than those for the calves fed the weekly prepared corn oil filled milk. These differences in fat digestibility, however, were not statistically significant (2).

Despite the beneficial effects of daily preparation noted here, no appreciable improvement was noted in the average daily gains made by these animals, as compared to those of subjects receiving the corn oil filled milk prepared at weekly intervals. The average daily gain at the end of 42 days of experimental feeding for the daily prepared group was 0.92 lb., as compared to 0.86 lb. for the calves on the regular corn oil filled milk regime. Since the animals fed the daily prepared corn oil filled milk were on the experiment for only 42 days, owing to a lack of time and funds, insufficient tocopherol determinations were made to ascertain the effects of this diet on blood plasma tocopherol values. No necropsy studies were performed on these calves, for the same reason. The results obtained by feeding a daily prepared corn oil filled milk indicate that peroxide levels greater than 5.0 meq. of O_2 per kilogram of fat, as measured by the method of Stine *et al.* (26), or factors associated with peroxide levels of that magnitude, were undoubtedly responsible for the pronounced diarrhea and low dry matter digestibility observed in calves fed the weekly prepared ration. The data suggest, however, that the severe diarrhea and low dry matter digestibility per se were not responsible for the poor gains made by the corn oil filled milk calves.

Other results obtained in this investigation also suggest that the feeding of a corn oil filled milk to calves depresses growth in some manner (1). Average gains made by animals fed corn oil filled milk were slightly less than those of subjects fed a calorically subnormal diet of skimmilk. Similarly, average gains for calves receiving the fortified skimmilk ration containing 3.5% corn oil were somewhat lower than those of individuals fed the fortified skimmilk without additional fat. This situation is in sharp contrast to the markedly greater gains noted when 3.5% hydrogenated vegetable oil was included in the fortified skimmilk ration. The growth-depressing effect of corn oil appears to transcend the differences in digestibility presented in an earlier report (2).

Additional studies were made to obtain further evidence of a possible toxic effect resulting from corn oil feeding. One calf near death from corn oil feeding was placed on a ration consisting of equal parts of corn oil filled milk and whole milk. The feces of this calf appeared normal within five days and all signs of weakness disappeared by the end of a nine-day period. The animal

was sold on the market 32 days following the change in diet. Before the change, the average daily gain of the subject was 0.43 lb., as compared to 1.46 lb. on the ration containing equal parts of corn oil filled milk and whole milk.

In Figure 3 may be found the growth curves of two additional animals which were used to note the effects of the corn oil filled milk on the well-being of older dairy calves, and to obtain further information on the effects of replacing portions of the corn oil filled milk with other rations. Both subjects were Holstein bull calves weighing approximately 275 and 285 lb., respectively, when removed from a ration of skimmilk, alfalfa hay, and a concentrate mixture at 115 days of age. During a ten-day transitional period, these individuals received

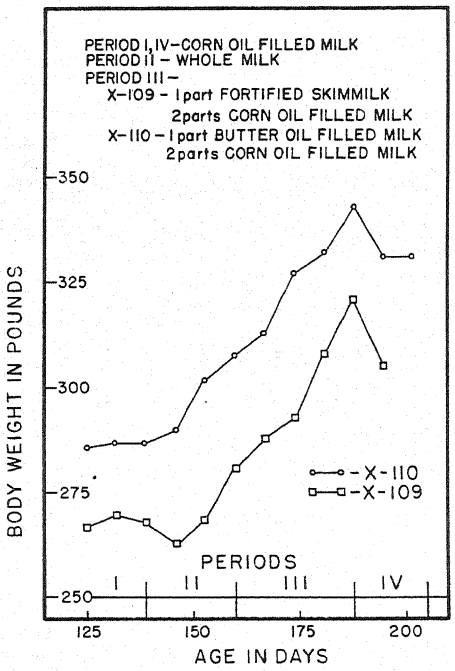


FIG. 3. Growth curves of two calves placed on the corn oil filled milk diet at 115 days of age.

only whole milk. By the end of this period, the weight loss due to the removal of fill and the somewhat drastic change in the diet had ceased. The calves were then placed on the corn oil filled milk ration. Following slightly more than 2 wk. of experimental feeding, the animals were exhibiting most of the symptoms already mentioned as occurring in corn oil filled milk subjects (1). The improved weight gains noted when only one-third of the daily allowance of the ration consisted of a suitable diet are shown in Figure 3. This improvement occurred in spite of the persistence of the diarrhea and low blood plasma tocopherol levels in both calves. At 190 days of age, both animals were again placed on the corn oil filled milk diet. A sharp decline in weight resulted and symptoms of muscular involvement appeared within seven to ten days. The physical

condition of both subjects had deteriorated within this period to the point where it was necessary to sacrifice them in order to insure accurate necropsy findings.

On the basis of the limited evidence presented in this paper and reports in the literature, it is felt that a considerable portion of the improvement noted in the physical condition and gains of calves fed as little as one-third of the diet in the form of a satisfactory ration, may have been due to a reduction in the intake of a toxic factor(s) present in the corn oil filled milk. Apparently, the tolerance of calves for toxic factors, arising from the use of highly unsaturated fats under these experimental conditions, is much lower than that of young pigs fed similar diets. McConnell (20) and others have shown that the use of highly unsaturated vegetable oils in formulas for baby pigs gives results comparable to those obtained with hydrogenated vegetable oils and animal fats. Data reported by others (4) also suggest that the length of the storage period for filled milks may influence the condition of calves fed such rations. Fewer toxic effects also were reported by Iowa workers (14) when the level of highly unsaturated vegetable oil in the diet of calves was reduced from 3 to 2% of the ration.

The findings reported here suggest that growth may be depressed by feeding a corn oil filled milk to dairy calves. Such a suggestion is in general agreement with evidence cited by Burr and Barnes (8), as well as by Quackenbush (23), that oxidized or rancid fats exert a direct toxic effect which is manifested in the body tissues. It has been demonstrated by contemporary workers (13) that various substances having conjugated double-bond systems in common aggravated dermal lesions and decreased growth in fat-deficient rats. It is unfortunate that the effect on weight gains was not studied with a corn oil filled milk having a peroxide content as low as that of the butter oil or hydrogenated corn oil filled milk rations which produced satisfactory gains.

ACKNOWLEDGMENTS

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SOME EFFECTS OF FEEDING VARIOUS FILLED MILKS TO DAIRY CALVES. IV. NECROPSY FINDINGS, ELECTROCARDIOGRAPHIC STUDIES, AND CREATINURIA RATIOS[1]

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SUMMARY

Gross and microscopic lesions were demonstrated in the cardiac and skeletal muscles of calves fed corn oil or lard filled milk. Such lesions were also found in animals receiving a similar ration containing butter oil, which had been prepared from oxidized butter. Both oral and intramuscular supplementation of the corn oil and lard filled milk diets with high levels of tocopherol prevented the development of muscular involvement during the course of the experiment. No evidence of muscular involvement was encountered in calves fed a low-fat, tocopherol-poor ration for periods as long as 5.6 mo. The influence of certain fats in the diet on the production of so-called vitamin E deficiency lesions in cattle is discussed.

The microscopic lesions in cardiac and skeletal muscles are characterized in detail. In general, these lesions were typical of those described previously as occurring in white muscle disease or nutritional muscular dystrophy. Necropsy findings, other than muscular lesions, are also presented.

Marked changes occurred in the electrocardiographic pattern of subjects in the corn oil filled milk group. The factor(s) responsible for these gross alterations could not be ascertained from the available data. The determination of creatinuria ratios did not provide a means of following the development of muscular involvement in calves.

The symptoms of muscular involvement noted in corn oil filled milk calves (3) suggested the need for a detailed necropsy examination of the animals fed the various experimental diets. The electrocardiographic studies and creatinuria ratios reported here represent an attempt to follow the development of the muscular lesions, which were demonstrated in calves receiving certain filled milks.

Details regarding the feeding and management of the experimental subjects in this report have been presented previously (1, 2, 3).

EXPERIMENTAL PROCEDURE

Complete necropsy studies were performed on the experimental calves which were sacrificed at the end of 90 days of age or had died before completing the experimental period. The extremely poor condition of some animals required that these subjects be euthanized by electrocution to avoid post-mortem changes. It should be added that some individuals from several groups were not sacrificed

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until 5–6 mo. of age. All endocrine glands and other organs were weighed following the usual gross observations. Sections for microscopic examinations were taken from the various glands and organs, as well as from cardiac and skeletal muscles. Skeletal muscle sections were obtained from the rhomboideus and triceps groups of the thoracic limb; while others were taken from the semimembranosus, semitendinosus, and gastrocnemius of the pelvic limb. Heart sections were obtained throughout the walls of both ventricles, taking care to include sections of the Purkinje network. All sections were placed in a 10% formalin fixative. Except as noted otherwise, an H & E strain was employed, using Harris hematoxylin and acid alcohol eosin.

Electrocardiographic tracings were obtained from each calf at regular intervals, using a Sanborn Viso-cardiette. A pre-experimental record was made at from ten to 14 days of age. Final electrocardiograms were obtained at 90 days of age, or prior to death in animals unable to complete the experimental period. All tracings were taken with the calf in its usual pen. A standard recording speed of 25 mm. was employed. Since research conducted by Sellers *et al.* (19) with cattle indicated that Lead IV (precordial) appears to give the most standard and duplicable results from animal to animal, only this lead was recorded. The precordial lead is obtained by placing bipolar electrodes over the apex of the heart and the top of the right scapula. All measurements of the electrocardiograms were made as described by Burch and Winsor (10). Because recent studies by Sellers *et al.* (19) have shown that a single rather than bi- or triphasic pattern of the QRS complex often occurs in the precordial lead (Lead IV) of records obtained from cattle, the amplitude of the QRS complex was measured, rather than the potentials of the individual Q, R, and S waves.

Urine was collected from the calves for the periods during which the animals were in the digestion stalls (1). The total urine output was measured daily and composite samples taken for analysis. Creatine and creatinine values were obtained, using the procedure outlined by Folin (12). Creatinuria ratios were calculated by the method of Eppstein and Morgulis (11), as used by Hove and Harris (15).

RESULTS AND DISCUSSION

Necropsy findings. Except as noted otherwise, no appreciable differences in gland or organ weights were observed which might be attributed to the levels of fat in the diet, type of fat in the ration, solids content of the diet, or vitamin E status of the calves. All figures were on a wet-weight basis and were expressed as grams per kilogram of body weight. The variation among calves as well as diets appeared to be surprisingly small for most glands and organs. However, the values obtained for the thymus and spleen were somewhat more erratic.

It should be noted that several individual values on various diets differed markedly from the over-all or group mean. One animal out of 13 in the corn oil filled milk group had a heart value of 22.2 g. per kilogram of body weight, which is considerably above the over-all mean of 6.3. The heart values of other subjects on this diet approximated the over-all mean. Large thyroid figures

were obtained from four of the calves in the corn oil filled milk group. The thyroid value for these subjects averaged 0.9 g., as compared to the over-all mean of 0.3 g. per kilogram of body weight. All other thyroid values in this group of 13 animals closely resembled the over-all mean. An enlarged thyroid value also was found in one out of four individuals fed hydrogenated corn oil filled milk. It did not appear that gains were influenced by the presence of an enlarged thyroid.

Gross lesions, excluding those observed in muscle, were infrequently encountered. Those noted in the respiratory system were secondary pneumonia associated with various degrees of atelectasis, and consolidation. Such lesions were demonstrated grossly in six of 16 subjects in the corn oil filled milk group. Single instances were found in groups receiving corn oil filled milk with oral tocopherol supplementation, lard filled milk, and fortified skimmilk with corn oil. These findings substantiate the earlier observation (3) that calves fed corn oil were more susceptible to infection. Gross examination of the genito-urinary system revealed white foci on the kidney of two corn oil filled milk animals. The appearance of the kidney was typical of that described as white-spotted kidney and was not thought to be associated with a vitamin E deficiency. Very little, if any, depot fat was found in subjects receiving the various corn oil filled milk diets. The absence of noteworthy lesions in the intestinal tract is of considerable interest, in view of the marked diarrhea which persisted in these animals.

The microscopic lesions, other than those in cardiac and skeletal muscles, were not of great significance. Numerous instances of mild fatty metamorphosis of the liver were observed in animals on the several corn oil filled milk rations. One such case also occurred in the lard filled milk group. Microscopic examination of the various glands and other organs revealed no lesions which would suggest trends of dietary origin. The enlarged thyroids found in some individuals did not differ histologically from the normal-sized glands. No remarkable lesions were found in the enlarged heart noted in one of the corn oil filled milk animals.

When present, the gross lesions in skeletal muscles appeared as pale or white areas. In some cases, entire muscles of both the fore and rear limbs were affected; whereas, in others only scattered areas were found, usually in the rear quarters. The lesions always occurred bilaterally and were noted in calves fed corn oil, lard, and butter oil filled milks. The gross lesions, however, were observed in approximately only 40% of the subjects with demonstrable microscopic lesions. The extensive gross lesions shown in Figure 1 were found in a calf severely afflicted with muscular involvement. A pale or white area from this animal is contrasted with normal-appearing muscle taken from the same subject.

The gross lesions observed in cardiac muscle appeared as pale or white streaks in the ventricles, or as pale patches in the septal myocardium and papillary muscles. Gross cardiac lesions were not present in all calves having histologically demonstrated lesions. The degree of muscular involvement as well as other factors may have been responsible for this failure to observe gross lesions in all subjects, in which microscopic lesions were found in cardiac and skeletal muscles.

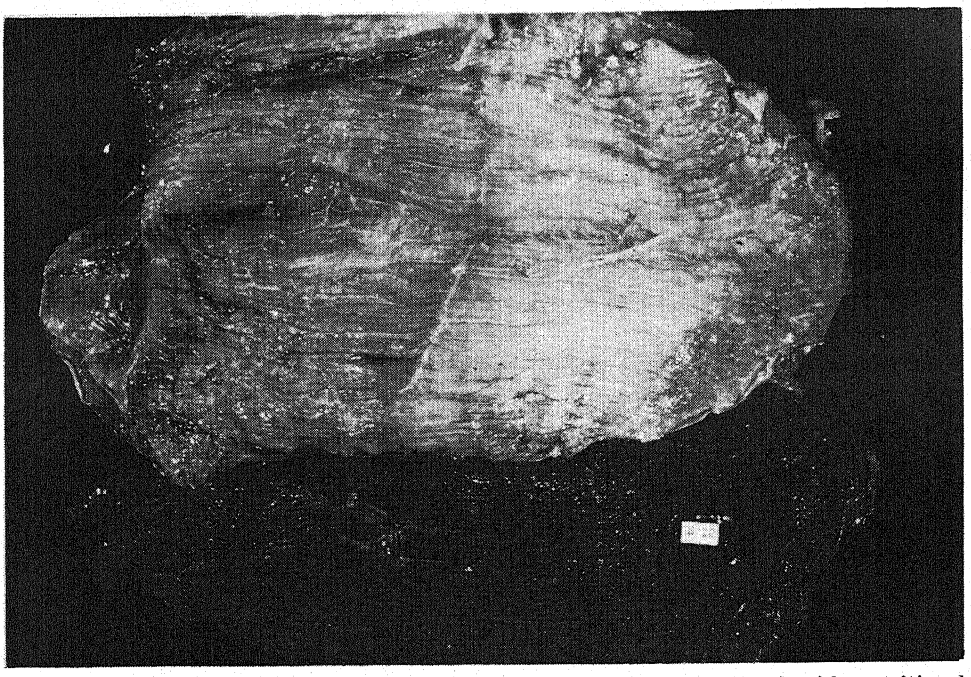


FIG. 1. Gross lesions in skeletal muscle removed from a calf afflicted with nutritional muscular dystrophy. Light area: affected muscle; dark area: normal muscle.

It should be added that the hearts removed from several individuals in the corn oil and lard filled groups were flabby.

The incidence of microscopic lesions in skeletal and cardiac muscles of calves on the various diets may be found in Table 1. Numerous animals in the corn oil and lard filled milk groups were afflicted with muscular lesions which could be demonstrated histologically. Similar lesions were also detected in two of the subjects receiving butter oil filled milk with a relatively high peroxide content (2). No microscopic lesions were observed, however, in animals fed a whole milk ration prepared and stored under similar conditions. The development of muscular lesions in calves receiving corn oil filled milk is of considerable importance. Previously, nutritional muscular dystrophy or white muscle disease has been produced experimentally only in calves fed lard or various fish oils (6, 7, 18). The presence of muscular lesions in the subjects receiving a butter oil prepared from oxidized butter is of equal significance. On each of the dystrophy-producing diets, no outward symptoms of muscular involvement were noted in several animals exhibiting microscopic lesions upon necropsy.

No muscular lesions were demonstrated in the corn oil or lard filled milk groups supplemented either orally or intramuscularly with the equivalent of 500 mg. of DL-alpha tocopherol or alpha tocopheryl acetate daily (2). Likewise, no muscular involvement was found in calves fed hydrogenated vegetable oil filled milks. Of greater importance is the finding that no muscular involvement

TABLE 1
Incidence of microscopic muscular lesions among the experimental calves

Diet	No. subjects autopsied	No. of animals with muscular lesions	
		Cardiac	Skeletal
Corn oil filled milk	16	4	12
Corn oil filled with oral tocopherol supplementation[a]	7	0	0
Corn oil filled milk with intramuscular tocopherol supplementation[a]	4	0	0
Hydrogenated corn oil filled milk	4	0	0
Hydrogenated cottonseed and soybean oil blend filled milk	2	0	0
Lard filled milk	6	3	3
Lard filled milk with oral tocopherol supplementation[a]	2	0	0
Butter oil filled milk	3	1	2
Fortified skimmilk	4	0	0
Fortified skimmilk with corn oil (including oral tocopherol supplementation)[a]	6	0	0
Fortified skimmilk with hydrogenated cottonseed and soybean oil blend	2	0	0
Skimmilk (including oral tocopherol supplementation)[a]	3	0	0
Whole milk	3	0	0

[a] See previous report (2) for details on tocopherol supplementation.

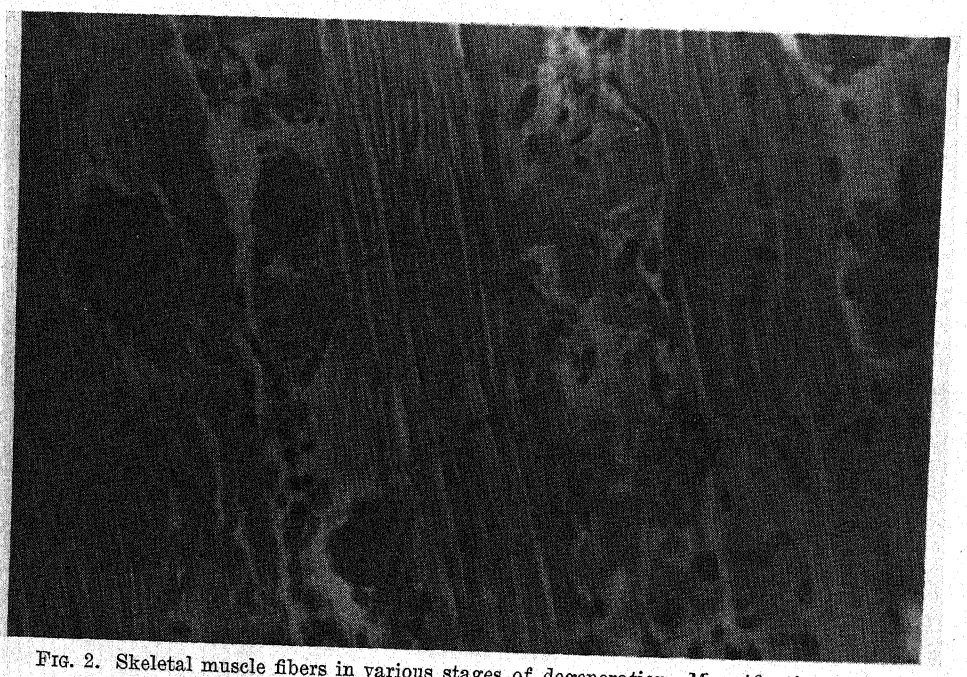


FIG. 2. Skeletal muscle fibers in various stages of degeneration. Magnification × 100.

or lesions occurred in subjects fed the low-fat and tocopherol-poor fortified skimmilk diet.

It appears that single muscle fibers and groups of fibers in certain areas are affected in the development of microscopic lesions in skeletal and cardiac muscle. Typical changes in an affected muscle may be found in Figure 2. Some fibers remain normal in appearance; whereas, adjacent fibers show signs of degeneration. As presented in Figure 3, the affected fibers appear to rupture at the Z line, forming retraction caps. Degeneration and necrosis apparently occur simultaneously with proliferation of the sarcolemmal nuclei and myocytes (Figure 2). The muscle fibers progressively show degeneration, necrosis, and fragmentation. Striations persist, however, and are easily recognized in remaining remnants. Within a short distance along a fiber the destruction varies in pattern from hyaline necrosis to atrophy. In atrophied areas the sarcolemma persists as a tube. The majority of the cells present in the later stages of degeneration appear to be sarcolemmal cells, along with a few lymphocytes. These cells are grouped around remnants of degenerating muscle fibers, forming pseudo-giant cells as shown in Figure 4. The centers of the pseudo-giant cells frequently contain

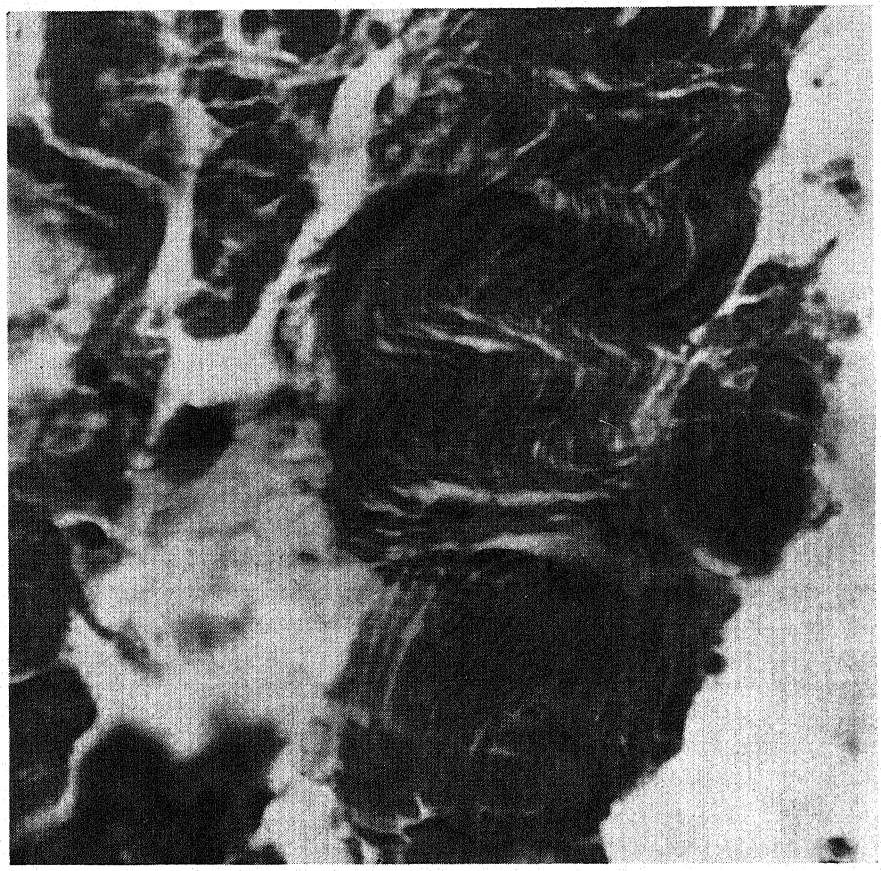


FIG. 3. Skeletal muscle rupturing at Z line. Magnification × 500.

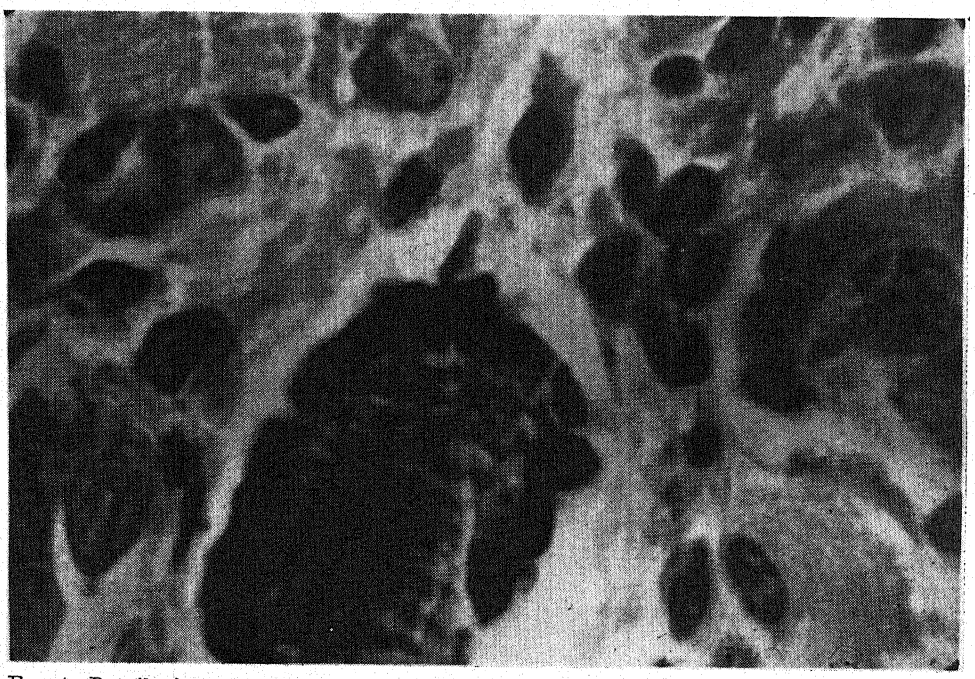


FIG. 4. Detail of a pseudo-giant cell as found in skeletal muscle lesions. Magnification × 800.

calcium, which can be demonstrated by staining with silver nitrate. Cardiac muscle changes as presented in Figure 5 were similar to those observed in skeletal muscle. However, they occurred less frequently.

The microscopic findings reported here are in general agreement with those presented by other workers. MacDonald *et al.* (16) as well as Vawter and Records (21) mention the presence of polymorphonuclear leukocytes. None was found, however, in this investigation. The lesions observed in the present study are in close agreement with those reported by Safford, Swingle, and Marsh (18).

The mean blood plasma tocopherol values were relatively low for all groups in which muscular involvement was detected (2). Lard is known to be relatively low in tocopherol, and it has been suggested that the butter oil used in this study may have had a low tocopherol content, as it was prepared from oxidized butter. However, the tocopherol content of the corn oil diet should have been adequate, as mentioned previously (2). Relatively high peroxide levels occurred in the corn oil and butter oil filled milk diets which produced nutritional muscular dystrophy (2). The peroxide content of the lard filled milk ration, however, was slightly less than that of the hydrogenated corn oil diet upon which no muscular involvement was found.

The evidence obtained in this investigation suggests that nutritional muscular dystrophy or white muscle disease, as it has been produced experimentally in calves, may not be the result of an uncomplicated vitamin E or tocopherol deficiency. In this experiment, the condition was found only in calves fed a highly unsaturated fat (corn oil), or fats with a relatively low tocopherol con-

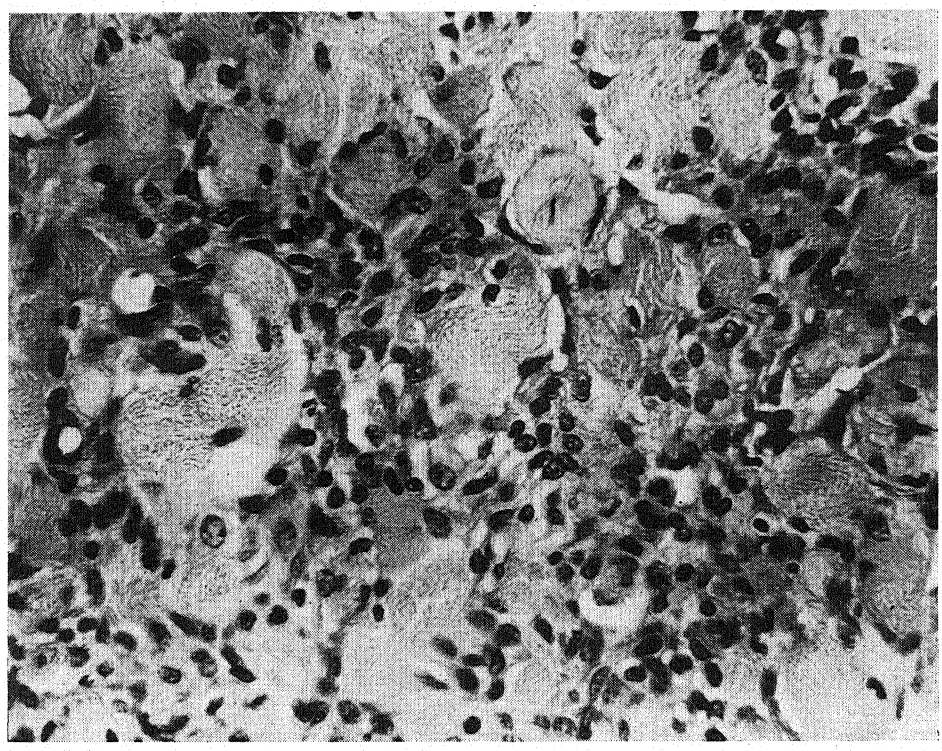


FIG. 5. Well-advanced lesion in cardiac muscle. Majority of nuclei are proliferating sarcolemma cells. Magnification × 400.

tent and an appreciable amount of unsaturated fatty acids (lard and butter oil). Despite extremely low blood plasma tocopherol concentrations, no evidence of muscular involvement was observed in animals fed a low-fat, tocopherol-poor ration (fortified skimmilk) to 5–6 mo. of age. Further support of the above suggestion is found in research conducted by contemporary workers. Blaxter *et al.* (6) concluded that the toxicity and nutritional muscular dystrophy noted in calves fed cod liver oil was due to the polyunsaturated fatty acid content of the oil and not to hypervitaminosis A or D. It should be noted that Brown (9) has demonstrated the presence of tocopherol in cod liver oil.

Singsen *et al.* (20) reported that chicks fed a basal ration containing only a small amount of tocopherol exhibited good growth, low mortality, and no encephalomalacia, in spite of low blood tocopherol concentrations. These workers stated that a low vitamin E condition per se does not result in encephalomalacia unless a stress condition, such as the presence of certain fats and oils, is placed upon the chicks. In view of present information, it is conceivable that the development of electrocardiographic changes, sudden deaths, and heart lesions noted in dairy cows fed a vitamin E–deficient ration (13, 14) may have been due to the presence of lard in the diet, rather than to a vitamin E deficiency per se. The earlier suggestion of Mason and Filer (17), that certain fats and oils in the diet influence the development of so-called vitamin E–deficiency

symptoms, by causing autoxidation to tocopherol during storage or digestion of the diet, and/or by increasing the tissue requirements for vitamin E in some unknown manner, remains plausible, in light of the findings reported here.

Electrocardiographic studies. Alfredson and Sykes (4) described the effects of heart rate on interval duration, as well as those variations within animals which may be attributed to serial tracings in normal cattle. More recently, Sellers *et al.* (19) obtained data concerning the individual variations in wave potentials as they occur in Lead IV (precordial) of tracings from normal dairy cattle. Thus, the changes and values obtained in this study were analyzed on an individual basis with these and other considerations in mind. As a result, significant changes which appeared to be of dietary origin were found only in the corn oil filled milk group. These changes consisted of increased potentials of the QRS complex and T wave, as well as an increased duration of the QRS interval.

Such gross alterations and others were noted in electrocardiograms obtained from three of five subjects fed the corn oil filled milk without tocopherol supplementation. Serial tracings of Calf E851 are shown in Figure 6. When compared with the pre-experimental record, the final record of this animal is characterized by increased potentials of the QRS complex and T wave, as well as by an inversion of the T wave. A marked widening of the QRS interval also occurred, and the P wave is not discernible in the tracing obtained two days before death. A reflection of the somatic tremors exhibited by the calf may be seen along the isoelectric line in the final record. Similar changes in electrocardiographic pattern were found in a tracing obtained three days before the death of Calf E857. A still greater disruption of electrocardiographic pattern was observed in the final record obtained from Calf E778. A suggestion of premature ventricular beats, with a tendency towards coupling, was noted in the final electrocardiogram of this subject. Other characteristics of the final tracing made four days before death included an alteration of the R waves and a widening of the QRS interval. All three of these corn oil filled milk calves were afflicted with symptoms of weakness and muscular involvement (3) at the time final records were obtained.

Calves E851 and E857 had final tocopherol levels of 65 and 175 μg. per 100 ml. of blood plasma, respectively. Cardiac and skeletal muscle lesions were demonstrated in both individuals. White streaks were observed grossly in the left ventricle of Calf E851. Microscopic examination revealed a proliferation of cardiac muscle nuclei and fibrosis in an area taken from the left ventricle. The only remarkable finding in the heart section obtained from E857 was an accumulation of fibroblasts around the inner Purkinje fibers. No data are available regarding the plasma tocopherol values or necropsy findings for Calf E778. The gross changes which occurred in Animals E851 and E857 appear similar to those observed by Bacigalupo *et al.* (5) in tracings taken within 24 hr. preceding the sudden death of vitamin E–deficient lambs.

Dietary factors presumably responsible for the changes found in electrocardiograms of calves fed the corn oil filled milk diet can not be ascertained

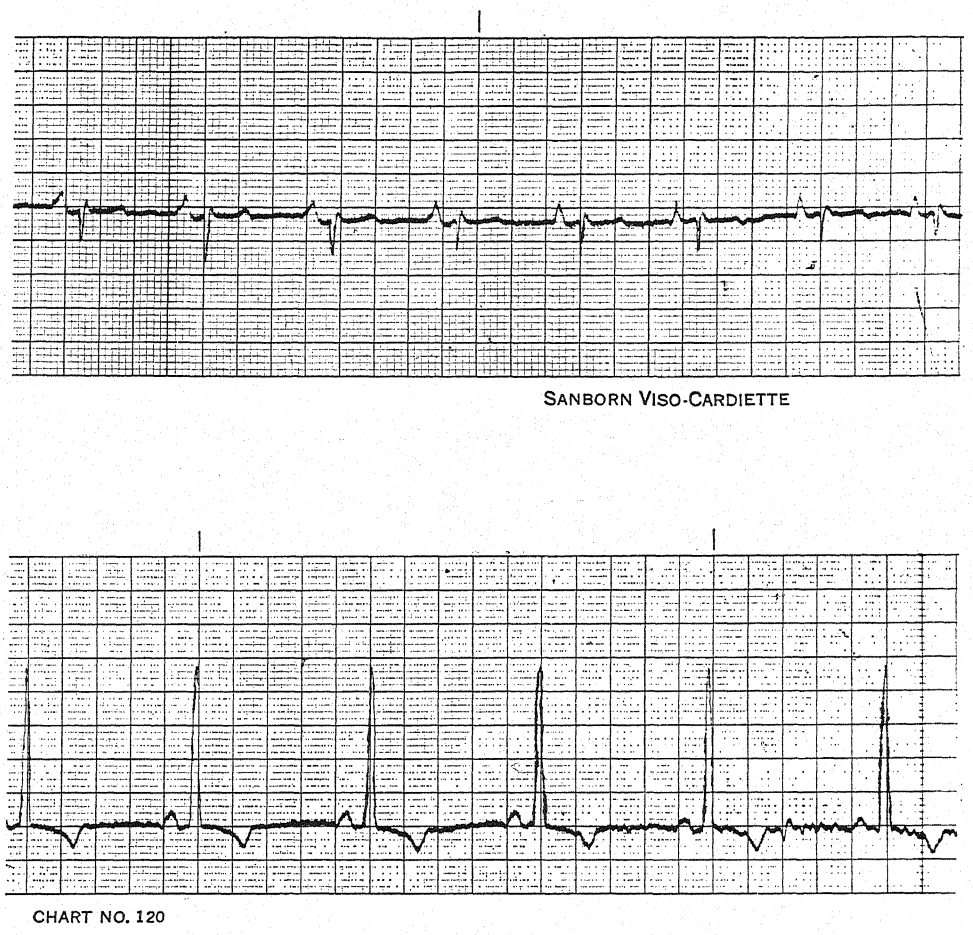


FIG. 6. Electrocardiographic changes in serial tracings obtained from Calf E-851, corn oil filled milk diet. Top, pre-experimental; middle, ten days before death; bottom, two days before death.

from the available data. Likewise, it can not be determined to what extent these changes resulted from the generally poor physical condition of the animals from two to four days prior to death, or were due to actual heart damage. It should be noted that no remarkable changes were observed in electrocardiograms of three additional corn oil and lard filled milk subjects in which cardiac lesions were demonstrated. A similar finding was reported by Bacigalupo *et al.* (5). These workers found that no significant alterations in electrocardiographic records occurred in some tocopherol-deficient lambs showing cardiac lesions upon necropsy.

Creatinuria ratios. An examination of the data pertaining to creatinuria ratios revealed that no marked changes occurred during the experimental period in all groups. The average daily excretion of creatine and creatinine, expressed as milligrams per kilogram of body weight, generally decreased on all diets during the course of the experiment. The absence of creatinuria in calves afflicted with nutritional muscular dystrophy is in agreement with results obtained by contemporary workers (7, 18).

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PASTURE FOR YOUNG DAIRY CALVES[1,2]

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SUMMARY

Two experiments were conducted with 32 dairy calves to study the value of pasture. In the first experiment, 22 Holstein calves were divided into three groups: barn-fed, pasture, and pasture plus hay free choice. The average daily gains were 0.97, 1.14, and 1.10 lb., respectively, to 60 days of age and 1.46, 1.67, and 1.70 lb., respectively, from 60 to 116 days of age. The differences in average daily gain were not statistically significant. The pasture group consumed 98.4 lb. less grain and the pasture-hay group 107.0 lb. less grain than the barn-fed group.

The results in the second experiment were similar to those in the first experiment, except that the Guernsey calves did not respond favorably.

Observations indicated that the pasture-hay calves consumed hay throughout the experiment even when an adequate supply of lush pasture was available. Calves having access to hay had a firmer feces and smoother hair coat than calves not having access to hay. Calves on pasture were observed to ruminate earlier than calves reared in the barn. All fecal samples were negative for parasite ova and coccidial oocysts. There are indications that consideration should be given to pasture mixtures for calves.

Although the rearing of calves on pasture has received considerable attention in New Zealand (7) and England (9), it has attracted little attention in America (6). In America, the usual practice is to wait until the calves are at least 6 mo. old before placing them on pasture. It would appear that this practice is based not upon experimental evidence but upon farmer experiences. Although very little is known of the ability of calves to utilize grass, there is evidence (2, 3, 5) which shows that 8- to 12-wk.-old calves can digest pasture as well as can mature cows. Another report indicates that the 3-wk.-old calf is able to digest pasture grasses as well as can older calves (8). This suggests that stomach capacity rather than digestion is the limiting factor.

Another reason for hesitating to place calves on pasture at an earlier age is their susceptibility to worm parasites. That parasites may not be a problem was suggested by the experiments of McCullough and Sell (6), who reported a low incidence of internal parasitism over a 4-yr. period. Even if parasites are present, it is unlikely that they will cause difficulty as long as an adequate supply of high-quality forage is available. Roy *et al.* (9) found that even when levels of infestation were above the values considered pathogenic for older animals, growth rate was not unduly depressed. In some cases, the best growth rates were obtained with calves showing the highest egg counts.

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The purpose of these investigations was to study the value and feasibility of using pasture for rearing young dairy calves under Indiana conditions, and to compare the results with calves reared under a conventional system.

EXPERIMENT I

Experimental procedure. In this experiment, 22 Holstein (16 bulls and six heifers) calves were assigned at random to one of three experimental groups: (1) barn-fed, (2) pasture, and (3) pasture-hay. The barn-fed calves were reared in a pole-type bulding with individual pens, feeders, and outside runways. The pasture and pasture-hay calves were placed on a pasture some distance from a barn. A pole-type shed covered with polyethylene plastic was used to provide shelter, and grain was available in individual covered feed boxes.

The pastures were divided into plots and the calves were rotated at intervals to provide them with a succulent, digestible forage at all times. The pasture forage consisted of an alfalfa-ladino-brome mixture at the beginning of the experiment. As the experiment progressed, ladino clover became the predominant forage.

The calves were obtained from the Purdue University and other state institution herds. All calves were left with their dams for variable lengths of time to obtain colostrum, then fed from open pails. All observations began when the calves were taken from their dams. The calves were not placed on pasture until at least eight days of age, but no later than 14 days. The daily feeding schedule is shown in Table 1 and the composition of the grain mixture in Table 2.

All calves were given access to grain three times daily. Hay was available to calves in Group 1 beginning at 15 days of age and to Group 3 when placed on pasture. Calves in Group 2 received no hay. Water was provided ad libitum by the end of the 2nd wk.

When the calves in Groups 2 and 3 were 61 days old, they were moved into larger pasture areas and the grain allowance was reduced to 2 lb. per calf daily until the experiment terminated at 116 days of age. Group 1 calves were kept in

TABLE 1

Daily feeding schedule

Age	Whole milk	Grain[a]	Hay
(days)		*(lb.)*	
0–2	On cow	....	....
3–7	6	....	....
8–28	8	3	Free choice[b]
29–35	6	3	Free choice
36–42	4	3	Free choice
43–49	2	3	Free choice
50–60	....	4	Free choice
61–116	....	2[c]	Free choice

[a] Indicates maximum allowed daily.

[b] Hay feeding began at 15 days of age for Group 1 calves and when placed on pasture for calves in Group 3. No hay was fed to calves in Group 2.

[c] Group 1 calves were continued on a maximum of 4 lb. grain daily to 90 days of age, then reduced to 2 lb. daily until the experiment was terminated.

TABLE 2

Composition of grain mixture

Ingredient	Per cent
Ground corn	58.0
Ground oats	25.0
Soybean oilmeal	15.0
Steamed bonemeal	1.0
Iodized salt	1.0
Total	100.0
Per cent protein	15.0

individual pens and allowed a maximum of 4 lb. grain daily until 90 days of age. They were then placed in large pens and group-fed 2 lb. grain daily and hay free choice until the experiment terminated. Calves in all groups were given free access to a mixture made up of two parts steamed bonemeal and one part iodized salt.

The calves were weighed at the beginning of the experiment and twice weekly until 60 days of age, then every other week until the end of the experiment.

Regular observations were made of the health and condition of the calves and, specifically, the incidence of diarrhea. Individual fecal samples were taken at the beginning and every 2 wk. during the experiment. Fecal egg counts for parasites were made according to the method of Stoll (10).

Results. The average daily gains for the calves to 60 days of age (Table 3) were 0.97, 1.14, and 1.10 lb., respectively, for the barn-fed, pasture, and pasture-hay calves. From 60 to 116 days of age the three groups of calves gained an average of 1.46, 1.67, and 1.70 lb., respectively (Table 4). Analyses of variance showed that in weight gains the differences were not statistically significant. Although the barn-fed calves made slower weight gains than either of the pasture groups, even these calves compared favorably to Holstein calves in the USDA Standard (Figure 1).

Feed consumption data are presented in Tables 3 and 4. The differences in amount of whole milk consumed are due to differences in age when calves were placed on experiment. Analysis of variance of the data in Table 3 showed that to 60 days there was a significant difference in grain intake between the three

TABLE 3

Average weight gains and feed consumption to 60 days of age

Calves	Barn-fed	Pasture	Pasture-hay
Number	7	7	8
Days on experiment	51.3	53.0	53.6
Days on pasture	.....	48.9	50.1
Growth rate			
Initial weight *(lb.)*	99.3	93.3	96.9
Average daily gain *(lb.)*	0.97	1.14	1.10
Standard error of mean *(lb.)*	.05	.07	.31
Feed consumption			
Whole milk *(lb.)*	236.0	247.7	251.0
Grain *(lb.)*	64.5	43.4	36.9
Hay *(lb.)*	44.5	.....	10.0

TABLE 4

Average weight gains and feed consumption from 60 to 116 days of age

Calves	Barn-fed	Pasture	Pasture-hay
Number	7	7	8
Growth rate			
Initial weight *(lb.)*	148.7	153.1	155.8
Average daily gain *(lb.)*	1.46	1.67	1.70
Standard error of mean *(lb.)*	.06	.28	.42
Feed consumption			
Grain *(lb.)*	187.3	110.0	107.9
Hay *(lb.)*	256.2	.....	20.5

groups. This occurred even though the amount of grain allowed was the same for all groups. The pasture group consumed an average of 21.1 lb. less grain and the pasture-hay group 27.6 lb. less grain than the barn-fed group. It was, likewise, noted that the average grain intake of calves in the pasture group varied from 22 to 74 lb. and from 16 to 69 lb. for the pasture-hay group. From 60 to 116 days (Table 4), the average grain consumptions were 187.3, 110.0, and 107.9 lb., respectively, for the three groups.

The pasture-hay calves consumed an average of 10.0 lb. hay to 60 days and 20.5 lb. from 60 to 116 days of age, as compared to 44.5 and 256.2 lb. by the barn-fed calves for comparable periods. This intake of hay by the pasture calves occurred even though young, lush pasture was readily available.

The pasture-hay calves produced a firmer feces and had a smoother hair coat than the pasture calves not receiving hay. The value of hay as an aid in the prevention of laxative feces was particularly noticeable when ladino clover was the predominating forage. At the conclusion of the experiment, the

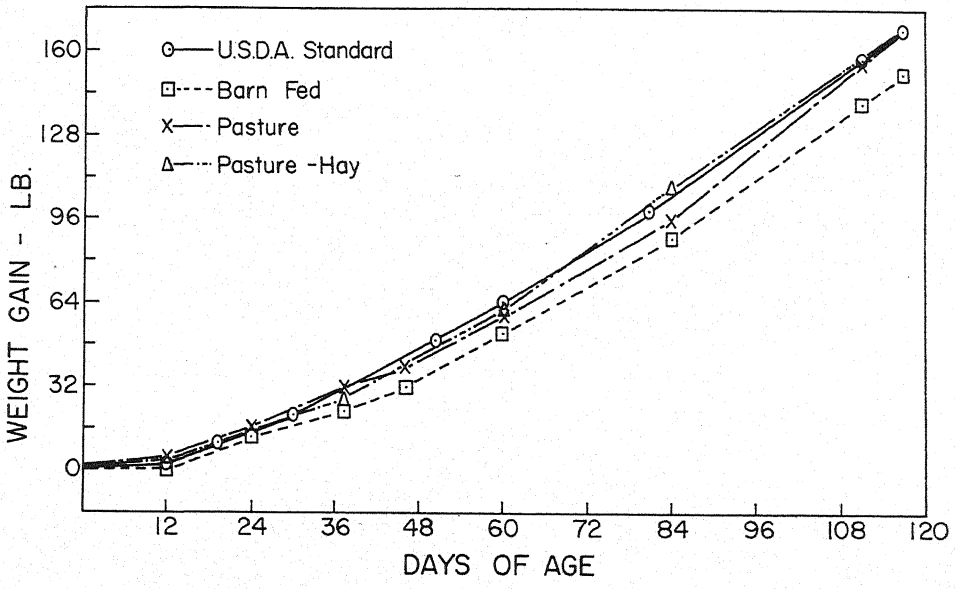


FIG. 1. Growth curves, Experiment I.

general appearance of the pasture-hay group was superior to the other groups, followed in order by the pasture and barn-fed groups.

Microscopic examinations of individual fecal samples for parasite ova and coccidal oocysts were negative throughout the experiment.

EXPERIMENT II

Experimental procedure. Six Holstein and four Guernsey calves were assigned to two groups: (1) alfalfa-ladino-brome pasture, and (2) alfalfa-ladino-brome pasture plus hay free choice. All calves were obtained from the Purdue University and other state institution herds.

The procedures used in this investigation were similar to those described in Experiment I, with the noted modifications. The daily feeding schedule (Table 5) allowed less whole milk than in Experiment I. It was necessary to

TABLE 5

Daily feeding schedule

Age	Whole milk		Grain[a]	Hay
	Holstein	Guernsey		
(days)	*(lb.)*			
0–2	On cow	On cow	....	....
3–7	6	6	....	....
8–14	8	6	3	....
15–21	6	6	3	Free choice[b]
22–28	4	4	3	Free choice
29–35	2	4	3	Free choice
36–42	2	2	4	Free choice
43–60	....	2	4	Free choice

[a] Indicates maximum allowed daily.
[b] Only Group 2 (pasture-hay) received hay.

modify the milk-feeding schedule for the Guernsey calves, to compensate for their lower body weights and the increase in incidence of diarrhea. Also, the Guernsey calves were fed milk to 60 days of age, as compared to 42 days for the Holsteins.

Results. The average daily gains for the pasture and pasture-hay groups were 0.80 and 0.81 lb., respectively, as shown in Table 6. The average daily gains of the Holstein calves were 0.97 and 1.07 lb., respectively, which is comparable to the gains of the Holstein calves in Experiment I to 60 days of age. In contrast, the Guernsey calves gained 0.55 and 0.41 lb., respectively, for the two groups.

During the experiment, the Holstein calves received an average of 176.0 lb. whole milk compared to an average of 211.5 lb. whole milk for the Guernsey calves. The Guernsey calves consumed an average of 23.5 lb. grain compared to 57.5 lb. by the Holstein calves. The calves in the pasture-hay group consumed an average of 5.5 lb. hay to 60 days. All fecal samples were negative for parasite ova and coccidial oocysts.

TABLE 6

Average weight gains and feed consumption to 60 days of age

Calves	Pasture	Pasture-hay
Number	5	5
Breed	3 H., 2 G.	3 H., 2 G.
Days on experiment	55.2	57.6
Days on pasture	51.2	51.2
Growth rate		
Initial weight *(lb.)*	81.6	68.4
Average daily gain *(lb.)*	.80	.81
Feed consumption		
Whole milk *(lb.)*	183.6	200.1
Grain *(lb.)*	44.4	43.6
Hay *(lb.)*	.....	5.5

DISCUSSION

That calves can make excellent use of good quality pasture at an early age was demonstrated in these investigations. These findings are contrary to the beliefs commonly held by many individuals, and are contrary to the usual recommendations, but they are in agreement with results reported by other workers (6, 7, 9), which suggest that calves can make excellent use of pasture at an early age.

Analyses of the growth curves in Figure 1 show that the barn-fed calves made consistently slower weight gains than the pasture groups and that the differences in favor of the pasture groups were still present at 116 days of age. The increased weight gains of the pasture groups are in agreement with the results obtained by other workers (6, 7, 9).

Th growth rates of the Holstein calves in Experiment I were comparable to those of Holstein calves in the USDA Standard (4). This is of interest, since the experimental calves received an average of 243.9 lb. whole milk to 50 days of age compared to whole milk until 4 wk. old, followed by skimmilk to 6 mo. of age by the calves as reported in the USDA Standard. That even less whole milk may be required was suggested by the results obtained with the Holstein calves in Experiment II, which consumed an average of 67.9 lb. less whole milk than calves in Experiment I but made comparable weight gains.

The ability of calves to utilize pasture was demonstrated by the pasture groups which made greater average daily gains than the barn-fed calves, with an average of 102.7 lb. less grain. The lower grain consumption was due to grain refusal. However, there was considerable variation between individual calves in grain consumption during the first 60 days. Inspection of the data indicated that there was no apparent correlation between grain consumption and weight gains, which suggests that there was considerable variation between calves in the amount of nutrients obtained from pasture. The increased grain consumption of the calves in Experiment II may have been the result of the lower amount of whole milk fed, animal variation, or the lower quality and quantity of the pasture during the experiment. Observations made during the experiment indicate that the poor results obtained with the Guernsey calves

in Experiment II were a reflection of their low grain consumption, or their unwillingness to consume sufficient pasture to overcome their lack of grain consumption.

Pasture consumption was observed to occur almost immediately when the calves were placed on pasture. One eight-day-old calf began consuming pasture immediately and was ruminating within 4 hr. after being placed on pasture. In general, calves on pasture began ruminating at a much earlier age than calves reared in the barn. At the present time there appears to be no satisfactory explanation or agreement as to when calves start ruminating. Reports range from as early as six days under barn feeding conditions (4) and seven days on pasture (7), to an average of 28.7 days under a barn-feeding system (1). Our observations are in close agreement with McMeekan (7) for calves reared on pasture and Ackerman *et al.* (1) for calves reared in the barn. The earlier observed rumination by calves on pasture may be the result of pasture consumption and its stimulatory effect on rumen activity. In a recent report, Preston *et al.* (8) suggested that the ability of the calf to ferment and digest grass dry matter was almost instantaneous and was dependent only on there being a suitable substrate in the rumen.

Observations made during both experiments suggest that calves on pasture have a desire for some coarse material which may be supplied as hay or pasture. Calves appeared to prefer brome grass, even when it was fairly mature, followed by alfalfa and then ladino clover. Ladino clover did not appear to be very palatable to young calves. Also, its consumption appeared to result in the production of a softer feces. The value of a dry feed for calves on pasture was indicated by the firmer feces and improved general appearance of the pasture-hay calves. The desire of calves for a dry feed while on lush pasture was also noted by Roy *et al.* (9).

Although it is recommended that young calves not be pastured because of parasites, the parasite counts were negative throughout both of these experiments. This suggests that the locations were free from parasites. Fecal samples taken from growing and adult animals in the Purdue University herd were also negative for parasites. It would appear that individual herds may be kept free of parasites if precautions are taken in bringing outside animals into the herd. It was suggested by Roy *et al.* (9) that even if parasites are present they may not affect the calf unduly, provided the pasture is plentiful and of good quality.

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THE STIMULATORY EFFECT OF SODIUM BUTYRATE AND SODIUM PROPIONATE ON THE DEVELOPMENT OF RUMEN MUCOSA IN THE YOUNG CALF

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SUMMARY

Twelve young dairy calves equipped with rumen cannulae were fed milk and housed in tie stalls with elevated floors to prevent bedding consumption. Sodium acetate, sodium propionate, sodium butyrate, sodium chloride, and glucose were administered over an 11-wk. period, to study their effect on rumen mucosal development. Two calves served as controls. Administration of solutions of sodium butyrate or sodium propionate caused marked development of the rumen mucosa, whereas the other materials caused relatively little growth. It is hypothesized that the growth of rumen papillae is probably a result of the metabolism of certain compounds by the rumen wall and/or their effect on blood flow in the rumen.

The newborn ruminant has a forestomach that is rudimentary both anatomically and physiologically. Contrary to early beliefs, the stimulus necessary to develop the forestomach tissues of young ruminants does not come entirely from the physical nature of the feed. Flatt *et al.* (7) reported that a low-fiber purified diet or a solution of the mixed sodium salts of acetic, propionic, butyric, and lactic acids caused the development of rumen papillae; whereas, coarse, inert materials, such as plastic sponges, were ineffective. The biochemical or physiological basis for the growth of rumen papillae following contact with the volatile fatty acids or a fermenting low-fiber diet is not clear.

In these experiments, stimulation of papillary growth using several pure, absorbable, and/or metabolizable compounds was measured.

EXPERIMENTAL PROCEDURE

Experimental animals. Twelve grade Holstein bull calves purchased at local auctions were fitted with cannula-type rumen fistulae (inside diameter ¾ in.) at 2 to 5 wk. of age, according to the method of Dougherty (4). They were confined in tie stalls equipped with elevated wooden platforms to prevent bedding consumption, and fed whole milk from nipple pails. The amount of milk fed was adjusted periodically in an attempt to keep the rate of gain uniform. Iodized salt and water were available free-choice. A mineral supplement and vitamin D were provided as previously reported (Warner *et al.*, 19). The calves were weighed weekly and records of feed consumption were kept.

Treatments. In addition to the basal milk allotment, eight of the calves were given a solution of either sodium acetate, propionate, butyrate, or chloride; and two were given a glucose solution via rumen cannulae. Two calves were randomly assigned to each treatment and two control calves received only milk.

Stock solutions of the sodium salts of the fatty acids were prepared, using analytical-grade reagents (Table 1), so that one liter of the solution contained an

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TABLE 1

Intakes, weight gains, and age at slaughter of the experimental animals

Calf No.	Treatment[a]	Age at slaughter	Total wt. gain	Total intake of		
				Milk	Test materials[b]	Test materials[b]
		(days)	*(kg.)*	*(kg.)*	*(kg.)*	*(M)*
3	Na Acetate[c]	109	34	586	15.4	257
4	Na Acetate	95	14	454	12.2	203
11	Na Propionate[d]	73	7	279	6.4	86
12	Na Propionate	102	34	516	16.0	216
7	Na Butyrate[e]	79	18	343	7.5	85
8	Na Butyrate	108	36	545	18.4	209
5	Glucose[f]	102	39	492	10.6	59
6	Glucose	102	36	518	10.1	56
9	Na Chloride	101	28	519	12.8	219
10	Na Chloride	103	41	595	8.8	150
1	Control	103	35	550	.....	.....
2	Control	104	41	529	....	.....

[a] All calves received the test materials for 11 wk., except Calves 4, 11, and 7. These calves died of pneumonia at the 9th, 5th, and 7th wk. of the experimental period, respectively.
[b] Amounts of sodium salts of fatty acids expressed as the acid.
[c] Analytical grade sodium acetate. Mallinckrodt Chemical Works, N. Y., N. Y.
[d] Sodium propionate N.F., E. I. duPont de Nemours & Co., Wilmington, Delaware.
[e] Analytical grade sodium butyrate. Amend Drug Co., N. Y., N. Y.
[f] Cerelose (dextrose). Corn Products Sales Co., Syracuse, N. Y.

amount of the salt equivalent to 250 g. of the respective fatty acid. These stock solutions were reduced to pH 6.7, using concentrated HCl. Before administration, each stock solution was diluted with warm water so that its concentration never exceeded 100 g. of free fatty acid per liter of solution. The amount of fatty acid salt administered was increased gradually during the experimental period.

A stock glucose solution was prepared at a concentration of 50% by weight. The stock solution was administered so that each calf on this treatment received 1.0 g. of glucose per pound of body weight per day.

The sodium chloride solution was slowly infused into the rumen during an 8-hr. period each day, according to Flatt's (8) method. This procedure was employed because typical symptoms of sodium chloride toxicity (Pistor *et al.*, 15), including severe tremors and incoordination in the rear quarters, were seen immediately after 3 liters of a 2% sodium chloride solution were poured into the rumen of a 90-lb. calf. The concentration of the infused solution was gradually increased from 0.9 to 2.4% during the 11-wk. experimental period.

The solutions (sodium chloride excepted) were poured into the rumens twice each day at the time of feeding. Table 1 summarizes the amount of each compound administered during the experimental period.

At the end of the experimental period, the calves were slaughtered. The digestive tracts were removed and the volume of water contained by the reticulo-rumen, filled to an overpressure of 1.5 in. of water, was determined in a manner similar to that used by Warner *et al.* (19). The reticulo-rumens were photographed under standardized conditions and the resulting photographs were used to give each rumen a papillary score, according to the technique of Flatt (8). A

4-in. square of the ventral portion of the dorsal cranial sac of the rumen was removed and manually separated into mucosa and muscle layers. These separated portions were dried at 90° C., allowed to come to equilibrium with the atmosphere, and weighed. The percentage of mucosa was then calculated on an air-dry basis. Following the removal of the 4-in. square of tissue, the rest of the rumen tissue was separated into mucosa and muscle portions. These portions, along with the reticulum and omasum, were dried in a Stokes freeze drier for 24-48 hr., using a temperature of 110° F. The dried tissues were equilibrated with the atmosphere, analyzed for fat-free dry matter, and expressed as grams of fat-free dry matter per unit of ingesta-free body weight (Table 2).

RESULTS AND DISCUSSION

The data obtained during the experimental period are presented in Table 1. All of the calves, except those that died from pneumonia, grew moderately well, as evidenced by the weight gains (Table 1).

The various treatments employed caused marked differences in rumen papillary development. The percentage of air-dry mucosa, the fat-free dry weight

TABLE 2

Data obtained after slaughtering the experimental calves[a]

Calf No.	Treatment	Body wt. (ingesta-free)	Rumen: Papillary score	Rumen: Mucosa[b]	Fat-free dry matter[c]: Rumen: Mucosa	Rumen: Muscle	Total tissue of: Reticulum	Total tissue of: Omasum	Reticulo-rumen volume[c]
		(kg.)		*(%)*	*(g.)*				*(liter)*
3	Na Acetate	74	3	50	73	63	32	38	15
4	Na Acetate	56	3	45	69	96	38	34	24
	Av. (A)			48	71	80	35	36	20
11	Na Propionate	53	5	57	70	78	43	31	....
12	Na Propionate	75	5	62	89	80	40	25	17
	Av. (P)			60	80	79	42	28	....
7	Na Butyrate	58	6	58	84	85	42	36	33
8	Na Butyrate	76	6	73	124	67	48	65	30
	Av. (B)			66	104	76	45	50	32
5	Glucose	80	4	37	42	44	22	35	17
6	Glucose	77	3	31	36	57	24	30	18
	Av. (G)			34	39	50	23	32	18
9	Na Chloride	68	2	40	38	69	24	21	23
10	Na Chloride	78	3	48	46	68	30	38	16
	Av. (S)			44	42	68	27	29	20
1	Control	79	1	30	24	40	17	17	17
2	Control	75	1	34	26	42	18	30	16
	Av. (C)			32	25	41	18	24	16
				B>A,S, G, C	B,P>S, G,C	N.S.	B>A,S, G,C	N.S.	
				P>A,G,C A>C	A>C		P>S,G,C A>G,C S,G>C		

[a] Data were subjected to an analysis of variance (16) and a multiple range test (5). All significant differences are at the 5% level of probability.

[b] Determined from a 4-in. square removed from the ventral portion of the dorsal cranial sac (air dry basis).

[c] Calculated to 100 kg. of ingesta-free body weight.

of rumen mucosa per unit of body weight, and the papillary rating were used as indices of mucosal growth. Figure 1 shows typical photographs used to arrive at the papillary rating for each rumen. Sodium butyrate and sodium propionate stimulated much more rumen papillary growth than did sodium acetate, sodium chloride, glucose, or the controls. The two aforementioned compounds gave extensive papillary development over a large area of the rumen, whereas sodium acetate, sodium chloride, and glucose gave very limited development only in the ventral part of the dorsal cranial sac, as did the mixture of volatile fatty acids employed by Flatt *et al.* (7). Sodium butyrate and sodium propionate also tended to stimulate more reticulum growth than did the other compounds tested. No statistically significant differences ($P < 0.05$) could be detected between any treatments in terms of rumen muscle or omasal growth.

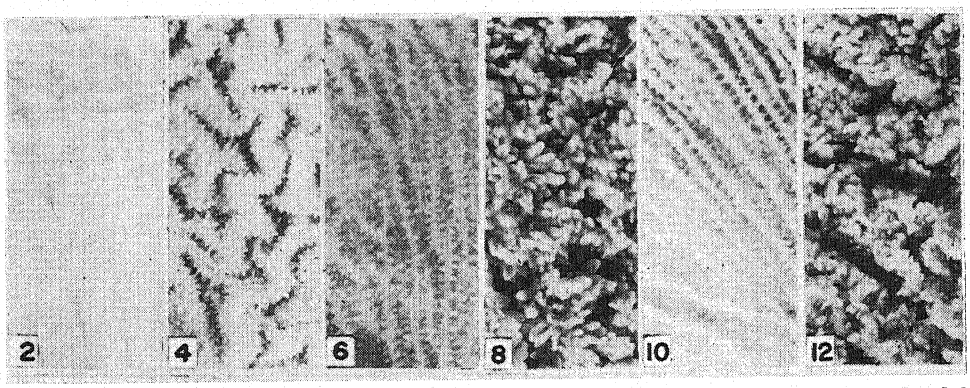


FIG. 1. Photographs of the ventral-most portion of the dorsal cranial sac, just caudal to the rumino-reticular fold. Photograph 2, control; 4, Na acetate; 6, glucose; 8, Na butyrate; 10, Na chloride; and 12, Na propionate. Papillary rating assigned to each photograph found in Table 2.

The volumes of water contained by the filled reticulo-rumens of the calves given sodium butyrate were decidedly larger than those of the calves on the other treatments, which were not appreciably affected by treatment. The physiological basis for this volume increase is not clear from the data obtained in this study.

Since the presence of absorbable fatty acids in the rumen has been shown to stimulate rumen papillary growth (7), it would appear reasonable to predict that the amount of rumen mucosal growth would be related to the degree of physiological or biochemical activity of the compounds as they passed through the membranes of the forestomach, viz., absorption, metabolism, and increased blood flow.

An examination of the literature does not reveal any studies which have duplicated the conditions of this experiment sufficiently to give an unequivocal explanation for the responses observed.

Much confusion exists in the literature that deals with the relative rates at which the volatile fatty acids penetrate the rumen wall (1, 2, 9, 13, 18). The

differences in relative rates of penetration as reported in the literature are not large and might well reflect differences in experimental technique. In studies with normal, conscious animals, in which the problem of interconversion of administered fatty acids within the rumen ingesta is eliminated, it has been shown that no preferental penetration of the fatty acids occurs on a molar basis (6, 17). Thus, one might expect the stimulation of forestomach mucosal growth to be similar for equimolar amounts of the fatty acids tested. As reported in this study, this does not appear to be the case. Calves 7 and 11, which succumbed to pneumonia before the completion of the study, had received approximately 85 *M* of butyric and propionic acid, respectively. Still, each of these calves showed much greater papillary growth (Table 2) than did either of the calves which had received more than 200 *M* of acetic acid.

Pennington (10) and Pennington and Pfander (11) have shown clearly that the butyric, propionic, and acetic acids are actively metabolized by the rumen epithelium in vitro. The relative degree of butyric acid metabolism was greater than it was for either propionic or acetic acids. In the experiment reported here, sodium butyrate afforded the greatest stimulation of rumen mucosa, while sodium acetate produced much less. The mucosal stimulation produced by sodium propionate was slightly less than butyrate, but it was much greater than that produced by acetate. Glucose has also been shown to be metabolized by rumen epithelium (12). If an active metabolism of materials from the rumen is necessary for papillary development, then glucose should have produced more than limited development. Whether the level of administration in this study was too low (Table 1) must be determined by further study.

Dobson and Phillipson (3) reported that increased fatty acid concentrations in the rumen caused increased blood flow from the rumen. Phillipson further reported (14) that the relative rate of increase was butyrate > propionate > acetate. The specific effects of an increased blood flow are difficult to predict, but it could certainly be expected to be associated with rapid tissue growth.

The data reported in this study strongly suggest that the growth of rumen papillae is most probably a result of the metabolism of certain compounds by the rumen wall and/or their effect on blood flow. Further research will be required to justify this concept.

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THE BIOLOGICAL HALF-LIFE OF L-THYROXINE AND L-TRIIODOTHYRONINE IN THE BLOOD OF THE DAIRY COW[1,2]

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SUMMARY

The biological half-life of thyroxine (t ½) is defined as the time in days for one-half of the biological activity of the hormone to be lost. An alternative measurement is the percentage turnover rate per day. By the injection of thyroxine-I^{131} into 16 dairy cows in which recycling of I^{131} was blocked by a goitrogen (thiouracil), the determination of the rate of disappearance of radioactivity from the blood permitted the calculation of t ½ and turnover rate. In 1957, during a period of average daily temperature of 47° F., the mean t ½ was 2.54 days and a turnover rate of 27.6% per day was observed. During a period of average daily temperature of 71° F., the mean t ½ was 2.65 days and a turnover rate of 26.7% was observed. In 1958, measurements were made at these two temperatures. Since these means did not differ significantly, it was concluded that seasonal variation in temperature did not influence thyroxine t ½ or turnover rate. The mean t ½ of 16 observations was 2.47 days and turnover rate 28.4% per day. No breed difference in t ½ in Jersey and Guernsey cows was observed. A significantly faster t ½ of 1.99 days and turnover rate of 35.3% was observed in a study of triiodothyronine. These data indicate that there is a build-up of thyroxine in the blood and total body space significantly higher than the daily thyroxine secretion rate.

A method for the estimation of the daily thyroxine secretion rate of dairy cattle has been described (3), and data have been presented concerning the range and mean secretion rate of cattle during various seasons of the year (5). It was shown that during the summer months in Missouri the secretion rate was reduced to about one-third the rate prevailing during the winter.

In the method proposed, it was pointed out that thyroxine and triiodothyronine as secreted are metabolized in the somatic cells of the body (muscle, brain, liver, and kidney) in which there is a further deiodination of the thyroxine molecule to triiodothyronine and other metabolic products. In the process of metabolism of thyroxine, the removal of one iodine molecule results in the formation of triiodothyronine, a biologically active compound equal to or greater than thyroxine (1). It has been suggested, in fact, that triiodothyronine is the biologically active compound at the cell level. The further metabolism (deiodination) of triiodothyronine results in biologically inactive compounds.

The iodine released is in the iodide form. It can be picked up by the thyroid gland or secreted in the urine. Thyroxine in the blood is also secreted in the bile and discharged into the digestive tract. Part of the thyroxine may be reabsorbed or excreted in the feces.

The rate of disappearance of thyroxine and triiodothyronine from the blood

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by deiodination or excretion is of great physiological importance, because it determines the duration of biological activity of these compounds in the animal body.

If the rate of disappearance were very rapid, the thyroxine secreted each day would be lost each day. The thyroxine secretion rate would then represent the effective blood thyroxine level. If, however, only one-half, one-third, or one-fourth of the thyroxine secreted each day were eliminated each day, then the residual biologically active thyroxine or triiodothyronine would accumulate in the blood and tissues and the biologically effective pool in the body would be proportionately greater than the daily thyroxine secretion rate.

It is now possible to study the rate of deiodination and elimination of the thyroid hormones by the determination of the rate of disappearance of I^{131} from the blood when l-thyroxine-I^{131} or l-triiodothyronine-I^{131} is injected into cattle. As the hormones are deiodinated in the tissues, the inorganic I^{131} returns to the blood and then could be reutilized by the thyroid gland or excreted in the urine. Since the reutilization of I^{131} would confuse the picture, it is necessary to block thyroid uptake by the use of a goitrogen.

By this technique, it is possible to determine the rate of deiodination and excretion of thyroid hormones. The biological half-life of a hormone may be defined as the time (in hours or days) required for one-half of the activity to be lost. An alternative measurement is to indicate the percentage turnover rate per hour or day. It indicates what percentage of the hormone secreted per day is metabolized and excreted each day.

The object of this report is to present data on the rate of deiodination and excretion of l-thyroxine and l-triiodothyronine in mature dairy cattle and to show the lack of effect of season (environmental temperature) upon the process.

EXPERIMENTAL PROCEDURE

Mature Guernsey and Jersey cows were maintained in a dry lot under uniform nutritional conditions throughout the year with free access to an iodized salt block. At the beginning of each experiment, the cows were injected intravenously (jugular vein) with 200 to 400 μc. of carrier-free l-thyroxine-I^{131} or l-triiodothyronine-I^{131}. To prevent thyroid uptake of the metabolized I^{131}, 24 g. of thiouracil was administered daily in a gelatin capsule (4). Blood samples were taken from the jugular vein each day following the injection of the hormone. Measurements of the radioactivity of the plasma were made in a National Radaic scintillation well counter (Model SA-2D). Conventional corrections were made for decay of the isotope and for background.

The residual radioactivity present in the plasma of each cow daily, when plotted on semilog paper, was found to form a straight line indicating the exponential nature of the rate of elimination of the thyroid hormones. The disappearance of radioactivity from the blood is assumed to represent the amount of thyroxine-I^{131} which has been excreted or deiodinated and the iodide-I^{131} formed excreted by the kidneys, since pickup of metabolized I^{131} by the thyroid gland was prevented by the use of a goitrogen.

The plot of the disappearance of I^{131} from the plasma was extrapolated back to zero time (time of injection) and biological half-time (t ½) of thyroxine was determined graphically (Figure 1) or from the slope of the regression line; t ½ represents the time required by the animal body to eliminate one-half of the thyroxine in the circulation.

The rate of metabolism of thyroxine per day was computed from the t ½ estimations by the following equation:

$$\text{Turnover rate per day } (\%) = \frac{100 \times ln_2}{t\ ½}$$

where ln is the natural logarithm.

RESULTS

To determine the rate of disappearance of thyroxine-I^{131} from the blood of dairy cattle, four groups of four cows each were studied during a period of 2 yr. The first study conducted in March, 1957, during a period of average daily temperature of 47° F., showed a mean t ½ of 2.54 days and a turnover rate of 27.6% per day (Table 1). In September, 1957, during a period of average daily temperature of 71° F., the mean t ½ of 2.65 days and a turnover rate of 26.7% was observed. The difference in the mean t ½ of these observations was not significant.

In March, 1958, during a period of average daily temperature of 53° F., a mean t ½ of 2.31 days and a turnover rate of 30.1% was observed; whereas, in

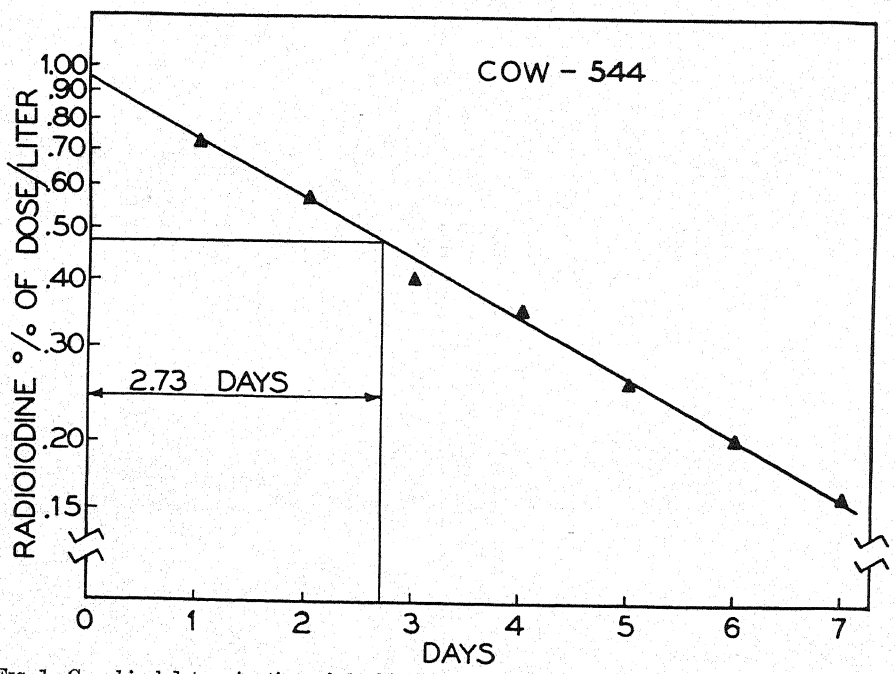


FIG. 1. Graphical determination of the biological half-life (t ½) of radiothyroxine in blood.

TABLE 1

The t ½ and turnover rate of L-thyroxine and L-triiodothyronine in the dairy cow

Breed	Cow No.	Date	Hormone	t½ Days S.E.	Turnover rate *(% per day)* S.E.	Daily temperature mean
						(° F.)
Guernsey	41	March, 1957	1-thyroxine	2.33 ± 0.053	30.0 ± 1.00	47
Jersey	544	March, 1957	1-thyroxine	2.71 ± 0.033	25.4 ± 0.62	47
Jersey	G41	March, 1957	1-thyroxine	2.25 ± 0.070	31.5 ± 1.32	47
Guernsey	38	March, 1957	1-thyroxine	2.87 ± 0.049	23.6 ± 1.03	47
Mean		March, 1957	1-thyroxine	2.54 ± 0.173 (1)	27.6 ± 1.86	47
Guernsey	41	Sept., 1957	1-thyroxine	2.45 ± 0.152	28.3 ± 2.87	47
Jersey	544	Sept., 1957	1-thyroxine	3.21 ± 0.070	21.6 ± 1.32	71
Jersey	G41	Sept., 1957	1-thyroxine	2.67 ± 0.028	25.9 ± 0.65	71
Guernsey	38	Sept., 1957	1-thyroxine	2.25 ± 0.026	30.8 ± 0.51	71
Mean		Sept., 1957	1-thyroxine	2.65 ± 0.206 (2)	26.7 ± 1.95	71
Guernsey	38	March, 1958	1-triiodothyronine	1.83 ± 0.105	37.9 ± 2.53	53
Jersey	158	March, 1958	1-triiodothyronine	1.88 ± 0.070	36.9 ± 1.67	53
Jersey	160	March, 1958	1-triiodothyronine	2.10 ± 0.065	33.3 ± 1.55	53
Guernsey	3596	March, 1958	1-triiodothyronine	2.14 ± 0.168	33.2 ± 4.03	53
Mean		March, 1958	1-triiodothyronine	1.99 ± 0.077 (3)[a]	35.3 ± 1.22	53
Jersey	348	March, 1958	1-thyroxine	2.43 ± 0.069	28.6 ± 1.13	53
Jersey	580	March, 1958	1-thyroxine	2.10 ± 0.068	33.5 ± 1.29	53
Jersey	594	March, 1958	1-thyroxine	2.42 ± 0.061	28.7 ± 1.16	53
Jersey	601	March, 1958	1-thyroxine	2.30 ± 0.052	29.6 ± 1.00	53
Mean		March, 1958	1-thyroxine	2.31 ± 0.082 (4)[a]	30.1 ± 1.15	53
Jersey	G41	July, 1958	1-thyroxine	2.49 ± 0.440	27.9 ± 10.52	73
Guernsey	38	July, 1958	1-thyroxine	2.69 ± 0.261	25.8 ± 6.24	73
Guernsey	41	July, 1958	1-thyroxine	1.97 ± 0.124	35.3 ± 2.98	73
Jersey	348	July, 1958	1-thyroxine	2.49 ± 0.090	27.8 ± 2.34	73
Mean		July, 1958	1-thyroxine	2.41 ± 0.229 (5)	29.2 ± 5.52	73

[a] Difference of mean (3) and (4) significant at $p < .01$; other means not significantly different.

July, with average daily temperature of 73° F., the $t\frac{1}{2}$ of 2.41 days and a turnover rate of 29.2% was shown. The difference in the mean $t\frac{1}{2}$ of these observations was also not significant. From these observations it may be concluded that the rate of disappearance of thyroxine-I^{131} from the blood of mature dairy cattle is not significantly influenced by seasonal variation in temperature at a time when thyroxine secretion rate is markedly influenced.

Since seasonal temperature did not influence $t\frac{1}{2}$ of thyroxine, the 16 observations were combined. The mean $t\frac{1}{2}$ was 2.47 days, with a mean turnover rate of 28.4%. Ten measurements on Jerseys showed a mean $t\frac{1}{2}$ of 2.50 days, with a mean turnover rate of 28.0%; whereas, six measurements on Guernseys showed a mean $t\frac{1}{2}$ of 2.42 days and a mean turnover rate of 28.9%. No breed difference between $t\frac{1}{2}$ in Jerseys and Guernseys is indicated.

Triiodothyronine has been shown to be secreted by the thyroid gland in small amounts and occurs in the blood in increased amounts as a result of the first step in deiodination of thyroxine. Four cows were injected with triiodothyronine-I^{131} during March, 1958, at the same time that the group was injected with thyroxine. This group showed a mean $t\frac{1}{2}$ of 1.99 days and a turnover rate of 35.3%. The difference in means of $t\frac{1}{2}$ between thyroxine and triiodothyronine is significant at $p < 0.01$.

DISCUSSION

The rate of metabolism and excretion of hormones is of great significance in the animal body for it measures the duration of effectiveness of the hormone upon the cells of the target organ or tissue which it influences. If the hormone is quickly metabolized (a few minutes) and eliminated as appears to be true of the steroid hormones (2), then the hormone blood level would be dependent upon hormone secretion rate from hour to hour. In the present study, it was shown that the mean $t\frac{1}{2}$ of thyroxine was 2.47 days, or that 28.4% of the injected hormone was removed from the blood each day. This means that the thyroxine secreted by a cow per day required 2.47 days for one-half of it to be eliminated from the blood and 2.47 days for one-half of the remainder. As a result of the relatively slow metabolism and excretion of thyroxine shown by this study, it appears that there is a build-up of thyroxine in the blood and total body space significantly higher than the daily thyroxine secretion rate. Using $t\frac{1}{2}$ of 2.5 days and turnover rate of 28.0%, it is possible to calculate the total blood and body thyroxine pool in animals secreting various levels of thyroxine per day by the equation:

$$E = \frac{X(1-K)}{K}$$

Where E is the total blood and body thyroxine pool, X is the daily thyroxine secretion rate, and K is the percentage turnover rate for thyroxine. Thus, in cattle secreting 5, 10, or 15 mg. of thyroxine per day/1,000 lb. body weight, the corresponding blood and body thyroxine pools would be 12.7, 25.5, and 38.5 mg., respectively (Table 2). The body thyroxine pool would be in equilibrium during periods of constant daily thyroxine secretion. However, with changing thyroxine

TABLE 2.

Thyroxine secretion rate, blood and body thyroxine pool, and effect of replacement therapy and stoppage in cattle

Daily thyroxine secretion rate/1,000 lb. body weight	Effective body thyroxine pool[a]	Thyroprotein replacement 15.0 g/day	
		Days to reach apparent equilibrium[b]	Withdrawal days to return to normal level[c]
(mg.)	*(mg.)*		
2	5.1	23	7.2
4	10.2	22	4.7
5	12.7	22	3.9
6	15.4	22	3.2
8	20.4	20	2.2
10	25.5	19	1.4
12	30.8	17	0.78
15	38.6	....	.....

[a] Calculated by formula $E = \frac{X(1-K)}{K}$ (see page 1610)

[b] Calculated by assuming a constant 28% turnover of thyroxine in the body.

[c] Calculated by the equation $E_n = E_o e^{-kt}$ where E_n = new elevated equilibrium of blood and body thyroxine pool under thyroprotein therapy; E_o = original endogenous equilibrium of total blood and body thyroxine pool; K = turnover % daily of thyroxine in body; t = time in days; e = base of natural logarithms.

secretion rate with temperature changes, the body thyroxine pool would change correspondingly. It will be noted, however, that the rise in the body thyroxine pool is slow during periods of increasing thyroxine secretion, whereas the decline is rapid during periods of decreasing thyroxine secretion.

This change can be shown in cows when replacement therapy with thyroprotein is practiced. The recommended level of thyroprotein-feeding is 15 g. per day to 1,000-lb. cows. It has been observed in our laboratory that 1 g. of thyroprotein fed is equal to the subcutaneous injection of 1 mg. of thyroxine. Thus, animals fed 15 g. of thyroprotein per day, whose daily thyroxine secretion rate was 5 mg. per day/1,000 lb. body weight, would require 22[3] days to reach apparent equilibrium of effective body thyroxine pool of 38.6 mg., which would be equal to the body pool of a cow secreting 15 mg. per day (Figure 2). On the other hand, upon the complete withdrawal of thyroprotein, the cow would return to the previous body pool of 12.7 mg. in 3.9 days. Similarly, cows secreting 6 mg. per day would return to the normal level in 3.2 days. The rapidity of decline is due to the destruction of the hormone at a constant rate while there is no endogenous secretion, since the thyroid gland is completely inactive during this period. In the case of cows fed thyroprotein for an extended period, the thyrotropin-secreting cells of the pituitary cease secretion and the thyroid gland would stop iodine accumulation and secretion of thyroxine. Upon the sudden withdrawal of thyroprotein and the rapid drop in the blood and body thyroxine pool, there would be a time lag in the resumption of TSH-thyroxine secretion for several days. In lactating cows this is shown by the precipitous drop in milk yield.

[3] Theoretically, equilibrium would be reached only at infinity. However, the magnitude of change after attaining 99% equilibrium is very small and if 99% of effective body and blood thyroxine equilibrium level is considered satisfactory, it will then take 13 days to reach equilibrium.

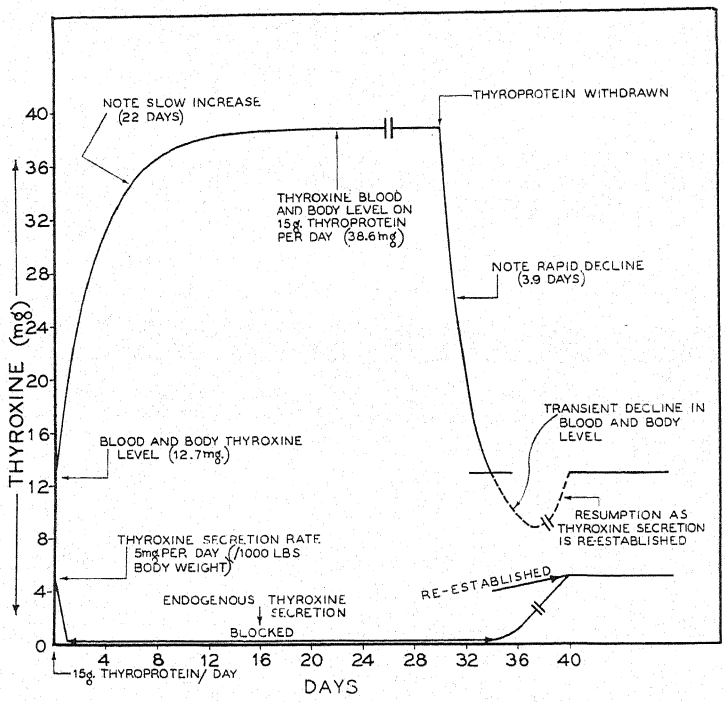


FIG. 2. Effect of feeding thyroprotein and sudden withdrawal on blood and body thyroxine level (pool). When 15 g. thyroprotein is fed to a cow secreting 5 mg. thyroxine per day, whose blood and body level is 12.7 mg., it requires 22 days to reach apparent equilibrium of the elevated effective body level of 38.6 mg. On complete withdrawal of thyroprotein, it requires only 3.9 days for the return to the previous thyroxine body level of 12.7 mg. However, when thyroprotein is fed for an extended period, TSH and thyroxine secretion is inhibited until the body thyroxine pool returns to 12.7 mg. Since time is required to re-initiate thyroxine secretion, the thyroxine body pool declines below normal, causing milk secretion to decline below normal. As TSH-thyroxine secretion is resumed, lactation increases to the normal level.

However, if the withdrawal of thyroprotein is gradual, as suggested by Swanson (6) and Swanson and Hinton (7), there would then be a gradual reduction in the thyroxine pool and time would provide for the gradual resumption of TSH and thyroxine secretion as the normal thyroxine secretion rate was approached.

These data are of significance also in understanding the pituitary thyrotropin–thyroid gland relation in estimating thyroxine secretion rate. As exogenous thyroxine is administered in increasing amounts below the normal thyroxine secretion rate level, the exogenous hormone would push the effective blood and body thyroxine pool above normal. The increased level of blood thyroxine would immediately act upon the TSH secretion rate and lower it slightly (Figure 3). A corresponding reduction in thyroidal-I^{131} discharge would occur. With each increase of exogenous thyroxine there would be a corresponding reduction of TSH and thyroidal-I^{131} release until, at the point of equilibrium of exogenous and

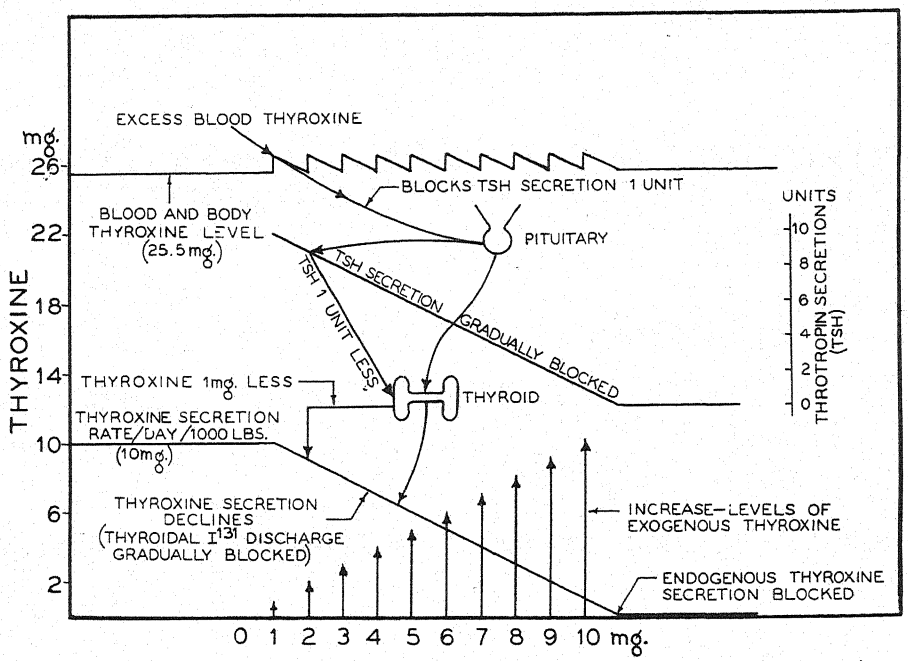


FIG. 3. Illustration of the principle of the determination of the thyroxine secretion rate by replacement therapy in a cow secreting 5 mg. thyroxine per day, with an effective body level of 25.5 mg. thyroxine. As exogenous thyroxine is administered in increasing amounts below the normal thyroxine secretion rate level, the effective blood and body thyroxine pool is elevated (i.e., slightly more than 25.5 mg.), which then acts upon the pituitary gland to reduce thyrotropic hormone secretion (TSH) slightly. A proportional reduction in I^{131} discharge from the thyroid gland will occur. The same phenomena will continue with increasing levels of thyroxine and, at the point where exogenous thyroxine equals endogenous production, all TSH and thyroidal I^{131} discharge will completely stop, indicating the blockage of endogenous thyroxine secretion. The dosage at which these phenomena occur is taken as the normal thyroxine secretion rate.

endogenous thyroxine, TSH and thyroidal-I^{131} release would stop.[4] This level of exogenous thyroxine, then, equals the endogenous thyroxine secretion rate. On the other hand, before this relationship was fully appreciated an injection of thyroxine in excess of the thyroxine secretion rate was administered, then reduced levels of thyroxine were administered. However, in such cases, the blocking dosages required time for their metabolism and elimination as indicated by these data and, as a consequence, the actual thyroxine secretion rate was underestimated, due to the time lag in thyroxine metabolism.

That the metabolism of triiodothyronine should proceed at a faster rate than

[4] It is generally recognized that the thyroid gland has a limited amount of autonomous functional capacity. That is, the thyroid gland may function slightly in the absence of TSH. Thus, slight thyroidal-I^{131} release may occur even after TSH blockage by thyroxine has been produced. The autonomous release of thyroidal-I^{131} seldom exceeds 5 to 10% of the previous count.

thyroxine is understandable, because the former compound has one less iodine molecule subject to deiodination. In spite of this difference in rate of metabolism, triiodothyronine appears to be equal to or slightly more active biologically than thyroxine. However, no direct evidence concerning comparisons of the two compounds in blocking TSH thyroidal-I^{131} release in cattle has been reported.

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TECHNICAL NOTES

METHOD FOR DETERMINING PRODUCT LOSS OF VACUUM-TREATED MILK

Vacuum treatment accompanied by flashing in nonsteam-injection systems for the removal of off-flavors from fluid dairy products results in concentration or loss of product. A comparison of Mojonnier total solids determinations on the original raw and treated milk samples has been used as a means of ascertaining these losses (2). The method is time-consuming and subject to the inherent errors of the Mojonnier test for total solids. Furthermore, the computed daily loss, based on periodic total solids determinations during processing, is not a true indication of the loss, because of variations in degrees of flash-cooling during a daily operation. The need for a practical method for determining product loss is apparent. This paper describes a method for determining product loss when fluid dairy products are treated in nonsteam-injection vacuum equipment.

METHOD

Vapors leaving the vacuum chamber on nonsteam-injection flavor improvement equipment are condensed by a water condenser located in the vapor line ahead of the vacuum pump. This is shown to the right of the broken line in Figure 1. Normally, the condenser water flows to the drain. The proposed method recirculates this water through a water collection system as noted to the left of the broken line in Figure 1. The water leaving the vacuum pump in a 1-in. stainless steel pipe flows through a sweet-water plate cooler which cools the water to approximately 35° F. This chilled water then enters a degassing chamber (located at a height above the vapor line condenser) through a 1-in. perforated stainless steel pipe and returns through ½-in. copper tubing to the vapor line condenser to complete the cycle. The excess water, resulting from condensation of the vapors leaving the vacuum chamber, overflows from the degassing chamber into an overflow cup and then into a condensate receiver on a scale. The weight of water accumulated per unit of time, divided by the pounds of milk processed per unit of time, multiplied by 100, results in the percentage of milk loss.

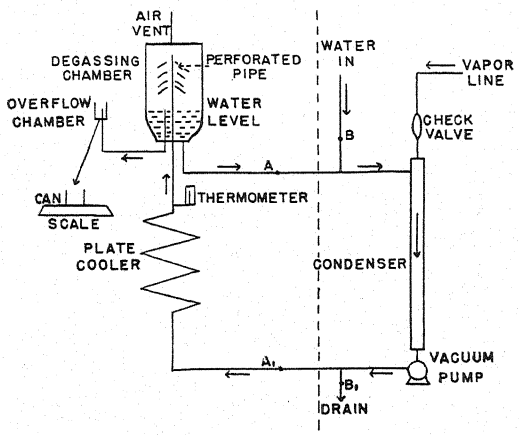


FIG. 1. Water collection system for estimating product loss resulting from vacuum treatment of milk.

The degassing chamber (19 in. by 9 in., tapering to a 4-in. diameter at the bottom) consists of a glass milk-receiver jar with a stainless steel plate at each end which allows for making the pipe line connections. The 1-in. perforated pipe which extends 16½ in. into the degassing chamber has ten sets of four ⅜-in. holes drilled 90° apart on the circumference. Each set of holes is ¾ in. apart, commencing 2 in. below the sealed upper end of the pipe. The air vent (1½-in. stainless steel pipe 6 in. long) prevents possible splash losses of water and pressure build-up by releasing the noncondensable gases.

The water collection system is started by opening Valves A, A_1, and B, closing Valve B_1, and starting the vacuum pump. When the system becomes filled to such an extent that water is flowing from the overflow chamber, Valve B is closed. Time should be allowed for the system to become stabilized so that a uniform overflow of water is established before water collection is started. Shut-off Valves A and A_1 allow for normal operation of the vacuum system with water flow to the drain.

EXPERIMENTAL PROCEDURE

A nonsteam-injection single-chamber vacuum unit,[1] located ahead of the final heater in a high-temperature short-time pasteurizer, was used to determine the reliability of the water collection system in comparison to Mojonnier total solids and theoretical calculations as a means for determining product loss resulting from vacuum treatment. Mixed raw milk, thoroughly agitated in a storage tank and balance tank, was pasteurized in a 3,000-lb.-per-hour pasteurizer at 172° F. for 16 sec. and homogenized at 2,500 p.s.i. after pasteurization and vacuum treatment. Ten trials, each representing a processing time of 1 hr., were made with a different degree of flash-cooling for each trial, so that flash-cooling increments from 5 to 18° F. were

[1] Vacu-Therm, DeLaval Separator Company, Poughkeepsie, New York.

studied during the trials. The average degree of flash-cooling for each trial was determined from observations (taken at four equally spaced intervals during each trial) of the temperature difference between the milk entering and leaving the vacuum chamber.

The weight of the overflow water collected in the water collection system during each trial, divided by the weight of milk processed, multiplied by 100, resulted in the percentage milk loss. The weight of milk processed per hour was calculated from the average weight of milk collected during four equally spaced 90-sec. flow times from the pasteurizing unit for each hour's run.

The latent heat of vaporization is a constant for respective pressures or vacuua, and radiation loss differences are minor with given equipment when operated at similar capacities. Therefore, the percentage of milk loss based on Mojonnier total solids determinations was calculated using a formula derived from total solids determinations in a previous experiment (2). This formula estimates milk loss from degrees of flash-cooling.

The theoretical percentage milk loss (radiation and convection losses disregarded) was derived from the data in steam tables (1), using the following formula, where h_g is the enthalpy of the saturated vapor and h_f the enthalpy of the saturated liquid:

$$\% \text{ milk loss} = \frac{\text{degrees of flash-cooling} \times 100}{h_g - h_f}$$

The method of least squares was used for determining the linear relationship between the degrees of flash-cooling and percentage milk loss. The standard error of estimate was used as a measure of accuracy of the line and the standard error of the slope as an indication of the accuracy of the slope of the line (3).

RESULTS AND DISCUSSION

A comparison of the water collection system with total solids determinations and theoretical calulations, as a means of determining product loss resulting from nonsteam-injection vacuum treatment of milk, may be noted in Figure 2. The upward trend in the percentage of milk loss, with increasing degrees of flash-cooling on a single vacuum chamber located before final heating, was expressed by the following equations, where Y represents the percentage of milk loss, and X the degrees of flash-cooling:

$$(1)\quad Y = -0.174 + 0.096\ X$$

for the water collection system; and

$$(2)\quad Y = -0.270 + 0.109\ X$$

for the total solids determinations. The standard error of estimate for the equations was, respectively, 0.03 and 0.19% milk loss. The standard error of the slopes was, respectively, 0.002 and 0.013% milk loss per degree of flash-cooling.

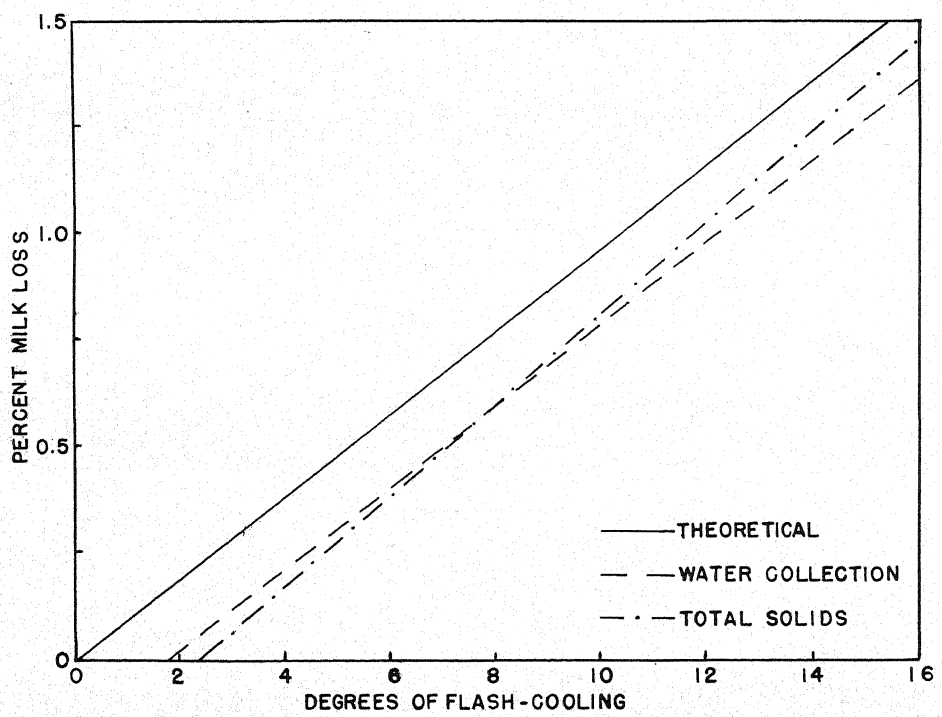


FIG. 2. Comparison of methods for estimating product loss resulting from vacuum treatment of milk.

The water collection line approximately parallels the theoretical line, but intercepts the Y axis at a lower point. This is to be expected since the theoretical line calculations did not include radiation or convection losses. The water collection line should converge on the theoretical line with an increase in the degrees of flash-cooling, since radiation loss will decrease with a decrease in the temperature of the milk leaving the vacuum chamber. However, this convergence is negligible and for practical purposes the water collection line is probably a valid estimate of milk loss. The water collection line does not agree closely with the total solids line. The total solids line converges on the theoretical line with increasing degrees of flash-cooling, but at a lower level of flash-cooling than can be postulated from radiation losses. A slight convergence as degrees of flash-cooling are increased is expected, but not to the extent indicated by the total solids line.

The water collection system appears to be more reliable than total solids determinations as a means of determining milk loss when processing milk in nonsteam-injection vacuum equipment for the following reasons: (1) The results more nearly approximate theoretical values; (2) milk loss estimates are more reproducible, as indicated by the low standard error values for the water collection system data presented in this paper; and (3) estimates of daily or hourly losses are more dependable, since the water collection system includes variations during processing of the milk.

Milk loss estimates can be approximated from degrees of flash-cooling (2), but the water collection system would be useful to dairy plants since it will minimize water usage as well as indicate more accurately product loss with non-steam-injection vacuum equipment. The system may also be used as a research tool in estimating losses with vacuum treatment and for collection of vapors removed by vacuum treatment for chemical analysis.

ACKNOWLEDGMENTS

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A SMALL-SCALE HEAT EXCHANGER CAPABLE OF HEATING MILK TO 325° F.[1]

For some time we have been interested in such problems as the effect of ultra-high temperatures on the physical and chemical properties of whole and concentrated milks, the thermal destruction of bacteria and bacterial spores, and heat-caused nutritional changes in dairy products. A need for a bench-size heat exchanger, capable of heating milk and concentrated milks to high temperatures in a short period of time, was the incentive to build such an apparatus. Certain performance requirements were decided upon. The heat exchanger should operate at the rate of 1 to 2.5 pt. per minute. It should be capable of heating milk from 40 to 325° F., and to do this in less than 3 sec. In addition, the apparatus must be simple to operate and yet dependable in its performance. A heat exchanger meeting these demands has been made and successfully operated. This unit is pictured in Figure 1 and diagrammed in Figure 2.

The following appears to be maximum heating conditions: pump rate of 2.4 pt. per minute, velocity of 19 ft. per second through the heater, heating time from 40 to 325° F. in 1.9 to 2.0 sec., and cooling time from 325 to 100° F. in 4 to 5 sec. Holding periods at the maximum temperature may be varied by changing the length of ¼-in. O.D. tubing connecting the heater and cooler. Each 4.08 ft. of holding tube will give 1-sec. hold. The accuracy of the controlled temperature appears to be within ± 1° F. Reduction of the pump rate proportionately changes these conditions. In addition to thermocouples (copper-constantan) located at the exit of the heater and cooler, additional thermocouples can be added before the heater and in the holding tube, so that a complete history of the heat treatment can be recorded on the 6-point recorder.

[1] This unit was built with financial support from the United States Steel Corporation.

The critical point in the construction of this equipment was the heater, where it was recognized that a velocity of at least 15-16 ft. per second was essential. Although there appeared to be several heater designs that might be used, the tapered spiral heater was selected to minimize the constriction effects that occur with small amounts of burn-on. The spiral grooves of the heater are 0.10-in. wide and 0.05-in. deep, with three revolutions per inch. The cooler originally served as a heater; however, some cross-over flow was found to occur between the spirals. This fact suggested the tapered design of the heater. Because 1.5-in. wall tubing was not available, the heater was machined from stainless steel castings. All product surfaces are stainless steel.

Pulsations were observed with the duplex pump, so a stainless steel accumulator employing a bladder was made and placed between the pump and heater. This has tended to eliminate the pulsations. To avoid flashing of the liquid in the heater, a back-pressure valve set at 75 p.s.i. was placed at the exit of the cooler. Without cooling water in the cooler, the system can be sterilized to avoid bacterial contamination.

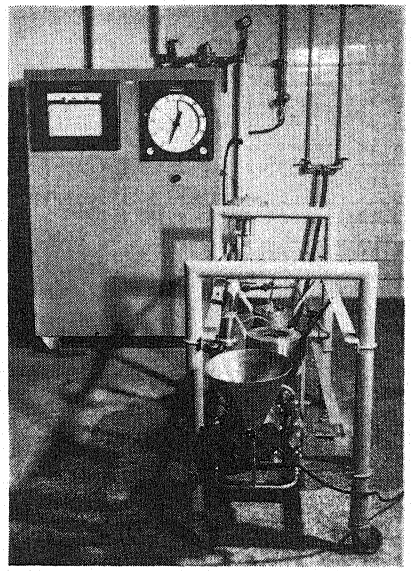


FIG. 1. Front-view photograph of the heat exchanger in operation.

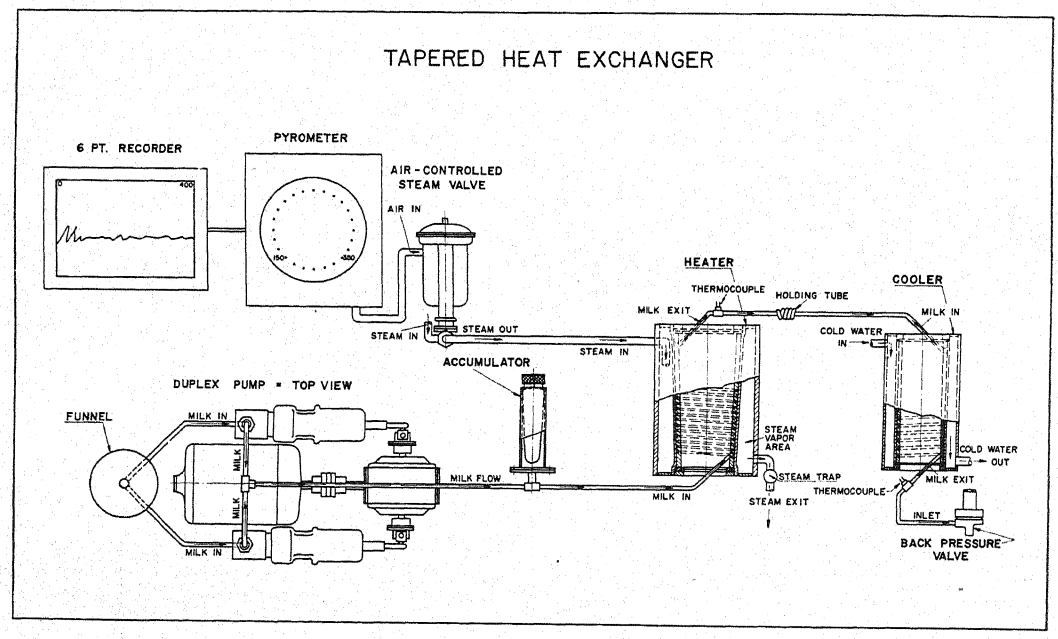


FIG. 2. Flow diagram of the heat exchanger.

The pyrometer is calibrated from 150 to 350° F. with a 2-degree scale. This is the temperature range of the heat exchanger. The 6-point multicolor recorder has a range of 0 to 400° F., with a two-speed drive.

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SPONTANEOUS LIPOLYSIS IN MILK THROUGHOUT THE STAGES OF LACTATION OF INDIVIDUAL COWS

Spontaneous lipolysis has been observed to occur in the milk from individual cows throughout lactation (1–5, 7, 9, 10–19, 21, 22), but its occurrence has been reported to be more frequent late in lactation (1, 3, 4, 5, 11, 14, 17, 19, 22). This type of lipolysis has been termed spontaneous by Tarassuk and Henderson (21), because it requires only cooling and aging to initiate lipolytic action. However, in some of these reports the methods used to evaluate lipolysis were lacking in sensitivity; therefore, total fatty acids liberated were not measured and in others such variables as the changing of feed were not eliminated. Recently developed tests for lipolysis or acid degree values (ADVs),[1] specifically the silica gel method of Harper *et al.* (8), recover the acids freed from the fat by lipolysis more completely than do some of the older methods.

It is well to remember that the terms rancidity and lipolysis are not synonymous. In the opinion of the author, the latter means fat hydrolysis at any level, whereas the former means fat hydrolysis to the extent that it can be detected organoleptically. The purpose of this investigation was to determine the extent of spontaneous lipolysis in a large number of milk samples, from a limited number of individual cows throughout one complete lactation with the silica gel method.

EXPERIMENTAL PROCEDURE

Four Holsteins whose lactation periods started between October 15 and November 10, 1957, were selected. Cows 2, 3, and 4 were in their first lactation and Cow 1 in the second. They were fed a ration of beet pulp, grain, grass silage, and alfalfa hay, but were not allowed access to pasture.

The cows were milked by machine and sampling was started three to four days after parturition. One-half pint portions were taken twice a week from the morning and evening milkings of each cow, and the samples were stored at 4° C. for 20–24 hr. The ADVs were determined by the silica gel method (8). All of the samples were checked organoleptically. Records were kept of production, butter fat content (Babcock), and unusual occurrences, such as illness and estrus.

RESULTS

As is illustrated in Figures 1 and 2, Cows 3 and 4 produced milk with greater susceptibility to spontaneous lipolysis in the later stages of lactation than did Cows 1 and 2. Each point on the graphs represents the average ADV of two morning and two evening samples. In general, except for Cow 1, the ADVs increased as lactation progressed. None of the samples from

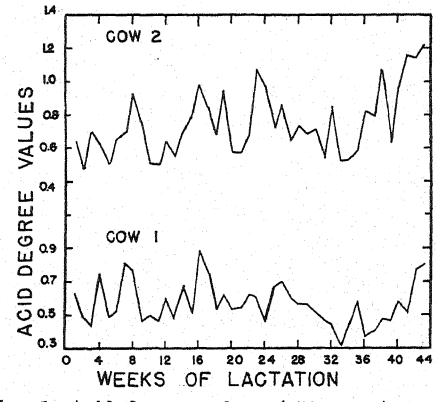


FIG. 1. Acid degree values (silica gel) of milk samples for complete lactations. Each point is the average of two morning and two evening samples. Acid degree value is the milliliters of 1 *N* alkali required to neutralize the acidity of 100 g. of fat.

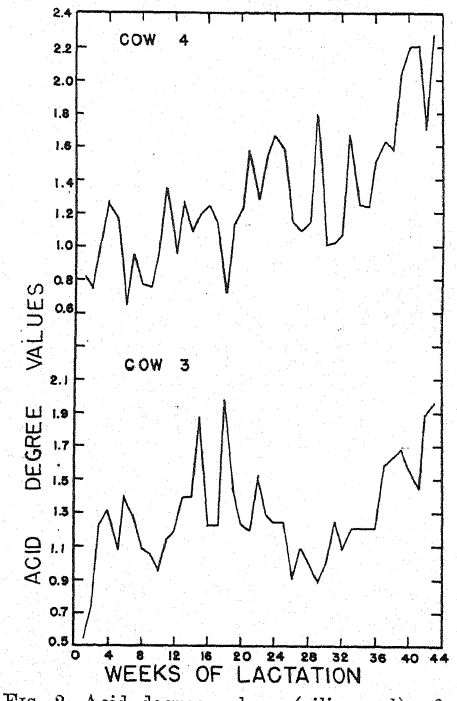


FIG. 2. Acid degree values (silica gel) of milk samples for complete lactations. Each point is the average of two morning and two evening samples. Acid degree value is the milliliters of 1 *N* alkali required to neutralize the acidity of 100 g. of fat.

[1] Acid degree value, or ADV, is the number of milliliters of *N* alkali required to neutralize the acidity of 100 g. of fat.

Cows 1 and 2 were rancid organoleptically. Nine of the 12 samples from Cow 3 which tasted rancid were taken during the last month of lactation and with Cow 4, 11 of 15 were obtained in the last month. All of the individual samples with a rancid flavor had an ADV of 1.90 or higher.

Table 1 shows that the average ADVs of Cows 3 and 4 were higher than the averages of Cows 1 and 2. As Table 1 illustrates, the average ADVs of the evening samples were higher than the averages of the morning samples, except for Cow 1. The differences were significant with Cows 2 and 3 when analyzed by the "t" test, but the average evening value for all cows was not significantly higher than the average morning values.

TABLE 1

Statistical data. Acid degree values (ADVs) of milk from cows throughout the stage of lactation

Cow No.	1	2	3	4
Av. ADV	0.57	0.75	1.25	1.28
Av. A.M. ADV	0.57	0.67	1.18	1.25
Av. P.M. ADV	0.56	0.80	1.31	1.30
"t" test				
A.M.-P.M.	0.1	3.522	2.263	0.9592
P	—	0.001	0.025	—

DISCUSSION

The data reported herein obtained by the silica gel ADV method confirm the work of previous investigators (5, 9, 10, 12, 13, 15, 18, 19), in that the susceptibility to lipolysis is subject to considerable variation from cow-to-cow as well as within cows over a complete lactation. Cow 1, which did not produce rancid milk and with the least variation in ADVs was a second-lactation cow, which is suggestive, but the limited number of cows studied does not permit conclusions.

The higher average ADVs of the evening milkings, as compared to the ADVs of the morning milkings, are difficult to explain, but for individual cows the difference may be valid, since a large number of samples were taken.

Figures 1 and 2 show that the weekly variation even in pooled samples was great, which illustrates the importance of cow individuality when dealing with spontaneous lipolysis. There are many points on the curves where the high ADVs can not be explained by stage of lactation or feeding, thus suggesting that unrecognized factors are operating. Tarassuk and Frankel (20) state that the lipase causing spontaneous lipolysis varies from a low concentration in normal milk to a very high concentration in naturally active milk. This may explain the individual variations noted in the present study. The explanations for the variations in lipase concentration are as yet unknown.

SUMMARY

The milk from each of four cows maintained under controlled conditions was checked for spontaneous lipolysis four times a week during a complete lactation by the silica gel method. The average acid degree values (ADVs) were: Cow 1—0.57, Cow 2—0.75, Cow 3—1.25, and Cow 4—1.28. The ADVs increased as lactation progressed with Cows 2, 3, and 4. Samples that were rancid organoleptically were produced only by Cows 3 and 4. Most of these samples were taken during the last month of lactation. There was considerable week-to-week and cow-to-cow variation. The average P.M. ADVs of Cows 2 and 3 were significantly higher than the average A.M. ADVS.

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OUR ASSOCIATION

ERRATA

CRITICAL FEATURES OF GOOD DAIRY FEEDING EXPERIMENTS

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Errata sheets for the mimeographed material distributed at the 54th Annual Meeting of the American Dairy Science Association are now available. In addition to a number of minor mistakes, a serious error was made in the construction of two of the tables in the Appendix of the handout. This error led to misleading answers for the problems in the Appendix that are based on the two tables.

In view of the serious nature of this error, the author would greatly appreciate having all who received the mimeographed material at the Annual Meeting write immediately for errata sheets.

BOOK REVIEW

THE UNITED KINGDOM DAIRY INDUSTRY. E. L. Crossley. Richard Clay and Company, Ltd., Bungay, Suffolk, England. 142 pp. 1959.

Professor E. L. Crossley of the University of Reading, with the help of 16 collaborators, has compiled an attractive book on the growth of the dairy industry in the United Kingdom during the past 30 years. This book is profusely illustrated with photographs, figures, and tables, a special feature being 22 colored pictures of the British breeds of cattle and of cheeses.

An attempt has been made to present a broad outline of the dairy industry in the United Kingdom, which consists of England, Wales, Scotland, and Northern Ireland, the Isle of Man and several other small islands. The United Kingdom has a land area of about 93,360 sq. mi., divided as follows: England 53.9%, Wales 8.6%, Scotland 31.9%, and Northern Ireland 5.6%. This area supports 51 million people, with 42 million in England, Wales 2.6 million, Scotland 5.1 million, and Northern Ireland 1.4 million. Out of this population there are 300,000 farmers and 698,000 agricultural workers. It is this land area, with the production of its workers, about which Professor Crossley tells his story of progress in the dairy industry. Both the production and consumption of milk have increased greatly. Traditional farm-house dairying has passed away, replaced with a modern industry of high technological development. The processing of fluid milk and distributing it to every family in the country are outstanding technological achievements of the dairy engineering industry, based upon research and education.

This small densely populated country has developed one of the great dairy industries in the world. Professor Crossley and his collaborators tell a most interesting story. This is a very attractive and readable book.

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APPLICATIONS AND LIMITATIONS OF QUALITY TESTS FOR MILK AND MILK PRODUCTS. A REVIEW[1]

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SUMMARY

Changes in milk production and handling practices of recent years have altered the types and behavior of bacteria in milk. There is a need for reappraisal of bacteriological tests currently in use. With growth of bacteria in raw milk virtually eliminated by efficient cooling, tests applied to the freshly taken sample are not always effective in detecting faulty production practices. Encouraging the growth of contaminants prior to testing increases the utility of the tests.

Longer refrigerated storage of pasteurized products is focussing attention on post-pasteurization contamination with psychrophiles. Here again, tests on freshly taken samples are much less useful than those made after these organisms have been encouraged to develop in the product.

Tests for specific groups of bacteria as indices of carelessness in production and processing probably will grow in importance. Coliform tests will have greater usefulness as efficient cooling hinders the growth of these organisms in raw milk, and as their significance in pasteurized milk is more fully appreciated. More emphasis on udder health may also be expected.

The term quality, as applied to milk and its products, embraces a variety of aspects. These include such diverse properties as freedom from dirt, antibiotics, off-flavors, pathogenic organisms, and abnormal numbers of body cells; evidence of cleanliness and care in production and handling as indicated by microbiological analysis; possession of desirable flavor and aroma, and of adequate amounts of those constituents of nutritional importance. Thus the meaning of quality differs from product to product. With milk itself, the bacteriological aspect has received the greatest attention. With butter and cheese, on the other hand, flavor is of far more importance. With ice cream, we are interested in both of these aspects as well as in composition. In dealing with the subject of quality tests, it was considered preferable to take each test in turn and consider its application, where appropriate, to milk and its various products. This review will not attempt to deal with the chemical and nutritional aspects of quality as these are extensive enough to warrant separate treatment.

MICROBIOLOGICAL TESTS

Interest in bacteriological testing of milk stemmed from the discovery that bacteria in milk could cause disease and spoilage. The first systematic investigation of a milk supply was that of Sedgewick and Batchelder in Boston in 1892 (144). From a public health standpoint the importance of bacteriological examinations was quickly recognized and gradually such examinations have become a regular practice. The need (135) for uniform procedures led the American Public Health Association in 1905 to appoint a committee to standardize methods. Studies by this and subsequent committees have resulted in successive editions of

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[1] Reprints available for 90 days after publication of this paper. For prices refer to page 1434 of the August issue of this JOURNAL.

Standard Methods for the Examination of Dairy Products (2), the 10th edition of which is currently being revised. In Europe there has also been interest in standardizing methods internationally; more recently, the International Dairy Federation has been active in this sphere (77).

With marked advances in the eradication of bovine tuberculosis and brucellosis, together with widespread pasteurization of milk, interest in bacteriological testing of raw milk has largely shifted from the disease aspect. Nowadays, such examinations are more concerned with obtaining an estimate of the degree of care taken in the production and handling of milk on the farm. Nevertheless, it should be remembered that conditions which allow the entrance and multiplication of large numbers of microorganisms may also allow the entrance and multiplication of pathogenic types.

The disease aspect is more important with pasteurized products, however. Recontamination with pathogens can have serious consequences. Direct testing for pathogens is impracticable, but testing to detect any recontamination after processing is now common.

High bacterial populations in milk may result from heavy initial contamination, as from neglected milking machines, from growth in the milk, or from both. Over the years, cooling procedures have greatly improved and, with the farm bulk tank, microbial growth is virtually eliminated. Consequently, cooling may mask careless production practices. This is causing concern to many milk sanitarians and there is a definite feeling that a reappraisal of testing methods and standards is desirable. Attempts to cope with this situation by promulgating stiffer standards—e.g., 50,000 per ml. (36)—are scarcely adequate, and involve setting up two different standards for acceptable milk.

Long ago it was recognized that the more carefully milk is produced, the less important cooling becomes. This is because the udder flora, which comprise the bulk of the bacteria in cleanly produced milk, grows best at body temperature and very slowly at temperatures below 15° C. (59° F.). In Britain, the importance of this has long been recognized. Official procedures (113) for testing raw milk call for it to be held at atmospheric shade temperature (averaging slightly under 60° F.) for 12 or 18 hr. before starting the test. Obviously, this is less satisfactory than a fixed temperature and would be out of the question where wider extremes of temperature are encountered. Recently, it has been suggested (90) that samples be given a preliminary incubation (P.I.) at 12.8° C. (55° F.) for 18 hr. before testing. At this temperature the udder flora fail to multiply. When counts made before and after P.I. are compared, little or no increase is found in carefully produced milks; others, even though initially low, may have increased over one hundred-fold. This procedure thus tends to show up those milks where cooling masks faulty production practices, and rightly puts the emphasis on clean methods rather than on efficient cooling. A somewhat similar procedure has been used with bulk tank milk in Scotland (30).

While bacteriological testing is essential in a quality control program, it is not enough in itself. Regular farm inspection is also considered essential. The work of the fieldman or sanitarian can be made much more effective by an adequate program of bacteriological testing. Both are indispensable.

QUANTITATIVE TESTS FOR TOTAL BACTERIA

The Standard Plate Count (S.P.C.). This method of assessing the bacteriological quality of milk was the first laboratory test devised for this purpose. Before pasteurization came into widespread use, there was a tendency to regard the plate count as a partial index of the freedom of the milk from pathogens. This view has not completely disappeared. Medical authorities in Britain still insist on 37° C. incubation for the plate count method, on the grounds that pathogenic bacteria will be more readily detected at this temperature. (It is usually recognized that the S.P.C. is of no value for this purpose.) Nowadays, it is more generally accepted that the chief value of the standard plate count (S.P.C.) (2) on raw milk is as an indication of the sanitary conditions of production and handling.

Although generally conceded to be the most precise method for assessing the bacterial population, the S.P.C. is not without limitations. No one medium incubated for a short time at a given temperature will bring out all the bacterial types present. Furthermore, colonies may represent single organisms or clumps of hundreds. The degree of experimental error inherent in the method is considerable (175), so that in comparison with chemical tests the plate count is a rather elastic yardstick by which to measure the bacterial content. This, unfortunately, is not always realized by administrators, and unwarranted significance is sometimes attributed to small differences in counts. There is also a tendency to consider the S.P.C. the sole criterion of hygienic quality, and to regard it as the standard against which all other tests for milk quality must be compared. Standardization of plating media, in order to ensure uniform productivity from different batches of media, has yet to be accomplished, although a synthetic reference medium has been developed (131).

Despite these limitations, the S.P.C. is generally conceded to be the most suitable method of examining raw milk with a low (e.g., less than 200,000 per milliliter) bacterial content. As quality improves, it is tending to replace other types of test. Some authorities consider it too laborious and expensive (59, 75); the United States appears to be the only country able to afford it for routine control of raw milk. Even here there is interest in simpler and cheaper procedures, such as the roll-tube method (5, 34, 75) and others (2, 67), especially for unofficial control purposes. In Britain, although the methylene blue test is the official test for routine control (113), the plate count is preferred for advisory purposes (19).

As a result of the studies at the Geneva Agricultural Experiment Station (130), the desirability of an incubation temperature lower than 37° C. gradually has been accepted. Dairy bacteriologists generally prefer 30 or 32° C. for a 48-hr. count. Some would like incubation for 72 hr. for pasteurized products (77). Public health laboratories, on the other hand, have generally preferred the higher temperatures used in their diagnostic work. To try to satisfy both groups, in 1948 incubation either at 32 or at 35° C. was made optional (2). S.P.C. at 32° C. is less likely to disagree with results of dye reduction tests, according to Harris *et al.* (65). Evidence continues to accumulate that 35° C. is too high

for certain types of bacteria (10, 11, 49), but there is still considerable resistance to adopting a lower temperature.

While the plate count is generally conceded to be the most suitable test for measuring the bacteriological quality of pasteurized products, about all it really tells when applied to freshly pasteurized products is how many thermoduric bacteria were present in the milk before pasteurizing. To this extent, a high count is useful in indicating neglected milking equipment, especially milking machines, the chief source of these organisms. In Europe, however, the primary interest appears to be in the keeping quality of the pasteurized milk or cream. Instead of making tests on the fresh samples, it is customary to hold samples at 5° C. for 24 hr., then at 17° C. for another 24 to 48 hr., before testing (16, 18, 125). After such treatment, a coliform test or a methylene blue reduction test is preferred to a plate count as correlating more closely with actual keeping quality at 17° C.

While the same incubation period is specified in *Standard Methods* (2) for both raw and pasteurized milks, the Committee set up by the International Dairy Federation (77) recommended an extra 24 hr. (72 hr.) for pasteurized milk. This is in recognition of the fact that many organisms which survive this treatment exhibit a long lag phase, resulting in failure to form discernible colonies in 48 hr. This is even more true with milk powder samples; after 72 hr., counts are often sharply increased, and colony size is much more satisfactory for counting (86). It is believed that *Standard Methods* should be revised to call for 72-hr. incubation for dry milks; the Committee of the International Dairy Federation (77) has recognized the need and has recommended incubation at 30° C. for five days.

In passing, it should be emphasized that a low standard plate count on a fresh sample is no guarantee of adequate keeping quality (87, 126). Milk may be a week or more old before it is consumed, and during that period has generally been exposed to temperatures which permit the growth of psychrophiles and some mesophiles. A freshly taken sample may show an extremely low S.P.C.—mostly thermodurics—yet, within a week at refrigeration temperature a slight contamination with psychrophiles may result in hundreds of millions per milliliter. As an index of plant sanitation, the S.P.C. made on samples after holding for five days at 7.2° C. (45° F.) is most effective (87); this procedure is much more sensitive than the coliform test, either before or after refrigerated storage. Samples with low counts after such treatment should have excellent shelf-life.

Other cultural methods. The S.P.C. requires considerable glassware, equipment, and media, and is expensive. Consequently, there is considerable interest in simplified methods, even where these may be less precise.

The Burri slant method (2, 28) employs a calibrated loop with which to deposit and spread 0.001 ml. milk over the surface of a hardened agar slope in a test tube. Although less accurate than the S.P.C., it is a useful screening test, especially where, as on the farm, no laboratory facilities are available. Modifications include the oval tube method for determination of thermoduric organisms (2, 119). The "Seeing Is Believing" test of Jamieson (2, 78), an offshoot of

the Burri slant method, can be used to advantage for swab tests on utensils, equipment, etc.

The roll-tube method has been proposed from time to time. With the development of improved apparatus, especially the Astell spinner, interest has revived, and favorable results have been reported (5, 34, 75, 140). The method is said to be used fairly extensively by milk plants in Britain. Heinemann and Rohr (67) have described a simplified procedure employing a loop and a screw-capped bottle which appears to have promise.

The membrane filter (MF) technique for total count yielded lower counts and was more cumbersome to carry out than the plate count method, according to Norwegian workers (57).

Surface plating techniques have been advocated from time to time. The latest of these is that of Mallmann and Broitman (105). None of them appears to have found favor for routine analytical work.

Direct microscopic count. This method (D.M.C.), described by Breed (2, 24) in 1911, has outstanding value in furnishing a bacteriological estimate within a few minutes. It also enables a count to be made of body cells (leucocytes, lymphocytes, etc.); this is especially valuable in indicating mastitis symptoms. With high-count milk the method, in the hands of trained technicians, can often furnish a clue as to whether utensil contamination or growth is responsible, provided the sample is from an individual can. It is also possible to preserve samples for later testing by adding formaldehyde (100). Despite these advantages, its popularity for controlling fluid milk supplies has been declining in recent years. In part, this is due to the trend to lower count levels, especially with farm bulk tank milks, necessitating the examination of many fields for quantitative determinations and, in part, to the realization that the D.M.C. grades high-count milk more leniently than does the S.P.C. (81). Black (21) reported the S.P.C. to give higher counts than the D.M.C., with counts exceeding 50,000 per milliliter; below this level, the reverse was true. The author has confirmed this. Another factor has been the growing recognition that many laboratories using the method lacked trained personnel and adequate equipment, especially illumination (21), and that often far too few fields were being examined (101). The effect of fatigue on qualified, conscientious technicians also has been belatedly recognized (99). The growing difficulty of obtaining suitable personnel, and their reluctance to undertake this work, must also be considered. While the D.M.C. is largely being superseded by the plate count for market milk, it is still a very valuable supplementary test here, and has considerable control value with the higher-count milk used in manufactured dairy products.

While the D.M.C. has been recommended in place of the S.P.C. for the control of pasteurized milk (111, 167), it has not been generally adopted for this purpose. More recently it is coming into use in the control of skimmilk powder (54). Here it gives valuable evidence of the past history of the product not obtainable from the viable count. Much more emphasis on adequate equipment and training and close adherence to *Standard Methods* is essential, however, if the method is to yield reliable results (54).

A modification of the D.M.C. for making counts of thermoduric bacteria was proposed (106), but does not appear to have been adopted. Poor agreement with the plate count has been reported (51).

"Little Plate" method. An attempt was made by Frost (56) to combine the advantages of the plate and direct microscopic counts. His little plate method involved growing the bacteria in a thin layer of nutrient agar on a microscopic slide, incubating in a moist chamber for 8-16 hr., then drying the film, staining and examining under a low-powered objective. The method is cheap and results are available earlier than with the S.P.C. Modifications have been published from time to time, but the method has failed to gain much support. Interest in its use as a screening procedure has recently been reported (44). It has also been proposed for making counts of bacteria, molds, and yeasts in butter (82, 114).

The methylene blue reduction test. The methylene blue reduction test (2), introduced in Denmark and Sweden around 1912, is probably the most extensively used bacteriological test, being widely employed in Europe, Australia, and New Zealand, as well as in North America. Its great advantages are its simplicity, cheapness, reproducibility, and rapid detection of poor-quality milks. Technically trained personnel are not necessary, and several hundred samples can be tested simultaneously. Thus, more frequent testing is possible.

Until recent years there has been good correlation between reduction times and plate counts, especially where the creaming error was minimized by periodical inversion of the dye-milk mixture during incubation. Wilson's (175) extensive studies were so favorable to the modified methylene blue test that in 1937 it officially replaced the plate count for the examination of graded raw milks in England and Wales (113).

Several factors have tended to distort the relationship between counts and reduction times. More productive media and lower incubation temperatures have increased the levels of plate counts. The proportion of thermoduric organisms has increased (152); these are slow reducers. Antibiotics in milk tend to slow down reduction. Psychrophiles, which sometimes comprise a high percentage of the flora (90), fail to grow at 35–37° C. Finally, with more efficient cooling, the bacteria are extremely dormant at the start of the test and substances inhibiting bacterial growth are conserved. The resulting prolonged lag phase so delays reduction that some high-count milks escape detection.

The creaming error, caused by the sweeping of varying proportions of the bacterial population to the surface with the rising fat globules (84, 175), has already been referred to. Wilson (175) recommended inversion of tubes every 30 min., to redistribute the bacteria and thus minimize this error, but hourly inversion is equally effective (83) and more convenient. The value of this practice is greatest with low-count milks; for milks reducing in 3 hr. or less, it is less important (83).

The practice in Britain of aging or storing raw milk samples at atmospheric shade temperature for 12 or 18 hr. before testing has already been mentioned. This stems from the 1920's, when regulations (112) governing the conduct of Clean Milk Competitions called for holding samples for around 24 hr. at atmos-

pheric shade temperature before subjecting them to analysis by the plate count. The weakness of this procedure lies in the wide range of temperatures encountered even in Britain. Thus, although the stipulated reduction time for the summer months is only 4.5 hr. as against 5.5 hr. in winter, summer samples show a much higher percentage of failures to conform than do winter samples. Furthermore, several workers have reported the grading to be too lenient in the winter months, a high percentage of samples with high bacteria counts not being detected (103, 166, 175). Storage at a definite temperature, e.g., 12.8° C. (55° F.) would seem more desirable; during cold weather it would allow the growth of saprophytic contaminants and result in shorter reduction times. Comparison of results from one season to another, and one year to another, also would be facilitated.

On this continent, the methylene blue test is rarely applied to pasteurized milk or cream. In Britain, where the interest is predominantly in keeping quality, Provan and Rowlands (136) recommended that the modified methylene blue or the resazurin test replace the plate count. After being held at 18° C. for 18 to 24 hr., the sample is incubated with the redox indicator at 37° C. for 0.5 hr.; samples failing to reduce the dye by this time are considered to have a keeping time in excess of one day at 18° C. Unfortunately, the official regulations (53) which followed specified holding at atmospheric shade temperature (not exceeding 18.2° C.) in place of 18° C., as recommended; methylene blue, rather than resazurin, was designated as the indicator, although Provan and Rowlands had favored the latter. In Britain, this type of test is still applied to ice cream also, but its suitability appears dubious. As Humphriss (76) states, "After all, the Public Health Officers, the manufacturer and the consumer are concerned with the wholesomeness of the product as eaten, and not after incubation for three-quarters of a day at warm room temperature."

As previously mentioned, some of the more progressive dairies in Europe use the methylene blue reduction test as a measure of keeping quality of pasteurized products (16, 18, 125). Their practice of holding samples at 5° C. for 24 hr. (to facilitate detection of oxidized flavor), then at 17° C. for 24 or 48 hr. before testing, avoids the variation in storage temperature of the British method and allows more satisfactory comparison of results from various periods. However, the coliform test is considered to be superior to the dye reduction test for this purpose (18, 125), particularly since rapid dye reduction is sometimes due to aerobic spore-formers which survive pasteurization (125).

For milk for manufacturing purposes, where standards are not as high as for market milk, the methylene blue reduction test is still widely favored in most countries. Unless and until standards for such milk are made much more stringent, it will doubtless continue in popularity. However, as cooling of such milk becomes more effective, its usefulness in its standard form will probably decline.

The resazurin test. As milk quality improves, reduction time increases, increasing the experimental error and making it difficult to complete testing in the ordinary working day. The introduction of the dye resazurin as a redox indicator to replace methylene blue (132) offered the advantage of earlier indication of Eh change, and aroused considerable interest. This was heightened

by the discovery (137) that resazurin was affected by the presence of excessive numbers of leucocytes etc. and thus could indicate the presence of abnormal (mastitic, late lactation, etc.) milk. This advantage is not possessed by methylene blue and is largely dissipated on prolonged storage. A number of modifications have been described, but only three of these have come into general use.

The 1-hr. test (137), modified from the original test by using 37° C. incubation in place of room temperature, was studied by various workers. In this test the degree of color change from the original blue through pink to complete decolorization is measured. While useful in reflecting abnormal udder conditions, it had limited value except where little quality-improvement work had been attempted; well-cooled milks containing excessive numbers of dormant bacteria often escaped detection (68, 69, 91). Where samples are aged before testing, as is officially required in Britain, this weakness applies only during the colder months. The pink test (84) which followed employed the full pink color as the end-point; this was reached in three-fourths of the time required to reduce methylene blue, but unfortunately the ability to detect abnormal milks was lost. A modification, somewhat more involved, has been proposed by Hempler (69), whereby abnormal secretion may be detected by a reading at 1.5 hr. and bacterial numbers by readings after 3.5 and 5 hr.

The triple reading test (2, 91) was developed to avoid the disadvantages of the 1-hr. and the pink tests. It employs a single end-point (Munsell 5P7/4) with readings after 1, 2, and 3 hr. This end-point is reached in one-half the time required for methylene blue reduction. Reduction beyond this end-point within the first 2 hr., followed by slow subsequent change, suggests reduction by leucocytes. The triple reading test has been recognized as a standard method (2) since 1948, and is widely used in Canada for market milk supplies. In order to distinguish milk of exceptionally good quality from that of only satisfactory quality, Hempler (68) has suggested a slightly stiffer grading with the triple reading test. He comments that the triple reading test, in addition to saving considerable time, "reflects the sanitary quality of the milk on a far broader basis than does the methylene blue test." Overby (127), after a study of various tests for the bacteriological quality of milk, also gave the preference to the triple reading test.

Of recent years, especially where farm bulk tanks are common, there have been criticisms that the resazurin triple reading test fails to detect an appreciable percentage of high-count milks. The organisms are so dormant that reduction is delayed appreciably. Here, too, preliminary incubation (90) is most useful in overcoming this dormancy, as well as in encouraging the growth of saprophytic contaminants. The poor agreement between S.P.C. and resazurin reduction time noted on fresh samples largely disappeared following P.I., and there was close agreement between gradings by these two tests (90). Unfortunately, reduction due to leucocytes is largely dissipated during P.I.; it is necessary to use a direct microscopic examination (2) or the Whiteside Test (117, 171), to detect abnormal milk.

Other forms of the resazurin test are employed in Britain. A 10-min. rejection test is applied to milk of doubtful quality (14). This test was selected during the war years as being the most satisfactory of eight methods compared for detecting milk unsatisfactory for pasteurizing, plate counts being of the order of many millions per milliliter (14). [In other countries, reliance is placed more upon a keen nose on the inspection deck; this appears to be a more delicate test (36).] Where cans are owned by the producer, it is also difficult to visualize cans of milk being held for over 10 min. awaiting results of the test.

In Scotland, a weekly temperature-compensated resazurin test is also officially required (42); the reduction time for acceptable milk varies with the mean atmospheric shade temperature during the preliminary holding period from 2 hr. at 40° F. to 15 min. at 61° F. and over. Where excessive leucocyte activity is suspected, a methylene blue test at 37.5° C. also is run. In Britain, grading is done using a Lovibond tintometer and standard color disks ranging from six for initial color to zero for complete decolorization. This method is considered to be more cumbersome and time-consuming than that using Munsell color standards (85) in test-tubes.

In any modification of the resazurin test, it is well to remember that 8% of the male population is color-blind; also that good illumination, preferably from a daylight type fluorescent lamp (40, 91), is highly important.

Golding's modification (61), wherein dye is added to sterile vials and dried down, is particularly useful for plants lacking adequate laboratory facilities, and for use by fieldmen testing suspected quarters for mastitis (108).

The use of another oxidation-reduction indicator, triphenyltetrazolium chloride, has been suggested (118). Unfortunately, it is extremely sensitive to light. Its usefulness appears to be confined largely to heavily contaminated milks, although it has been advocated for use in the detection of antibiotics and other inhibitory agents in milk (120), and in a keeping quality test for pasteurized milk (26, 41).

QUANTITATIVE TESTS FOR SPECIFIC TYPES OF MICROORGANISMS

Thermoduric bacteria. These organisms are sufficiently heat-resistant to survive ordinary pasteurizing temperatures and thus may be responsible for counts in excess of the legal limit for pasteurized products. They enter milk chiefly from the surfaces of inadequately cleaned milking and handling equipment and thus are an indication of insanitary conditions. Some sanitarians believe the thermoduric count is more useful here than the standard plate count (15). While the thermoduric count is officially determined by plating a suitable dilution of raw milk after being subjected to laboratory pasteurization (2), less expensive modifications have been sought. These include the oval tube method of Myers and Pence (119), streaking measured loopfuls of laboratory pasteurized milk on the surface of poured plates (2); the agar strip method (153), a version of the oval tube method, developed in Britain [it is preferred over the roll-tube method (5), as it requires no special equipment (47); counts by both methods were lower than by the standard plate count, but not enough to invalidate their

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successful use]; and inoculating melted agar in a test-tube or small bottle, holding at pasteurizing temperature for 30 min., then spreading the inoculated medium in a thin layer. The Astell version (5) of the roll-tube method has already been mentioned.

Incubation at 32° C. is preferable to that at higher temperatures; 37° C. is too high for some thermoduric species. British workers (37) recommended incubation at 30° C., for four days, but subsequently (47) reduced this to three days. While the results from these simplified methods are more variable than those by the official plating method, they are quite adequate for screening out those milks containing excessive numbers of these organisms. A standard of 10,000 per milliliter, commonly employed, seems unduly lenient.

Thermophilic bacteria. Organisms capable of growing at holder pasteurization temperatures were a serious problem when batch pasteurization was common. With the trend toward higher temperatures with HTST and UHT pasteurization, the interest has diminished. These organisms are detected by incubating plates at 55° C. for 48 hr. (2), by direct microscopic examination of smears (2), or by a dye reduction test (methylene blue or resazurin) incubated at 62–63° C. (94).

Psychrophilic bacteria. For the purpose of this review, psychrophilic bacteria are those able to grow actively at temperatures below 7.2° C. (45° F.). It should not be overlooked, however, that their optimum growth temperatures are generally around 25 to 30° C.; not one of 722 cultures grew at 35.5° C. (49). With milk being held longer at refrigeration temperatures from cow to consumer, opportunities for the growth of psychrophiles have increased greatly. Many of them are proteolytic and lipolytic, and are capable of inducing flavor and other defects in milk and milk products on refrigerated storage.

The current official procedure (2) for counting psychrophiles calls for incubation at 5° C. for seven days. Some claim that this low temperature is too selective. Milk and its products are frequently exposed to temperatures above 5° C.; some species capable of inducing spoilage can grow actively in this range. After seven days at 5° C., colonies are frequently so tiny as to be difficult to recognize; counts after ten days are frequently many times larger, while some workers (23) found 20 days necessary to obtain maximum counts. In Denmark, Hempler (70) prefers 7° C. for ten days;[1] this should be much more satisfactory.

Spoilage in pasteurized milk, cream, and cottage cheese is generally due to psychrophilic growth. Pasteurization destroys these organisms; their presence in processed products represents recontamination. Examination of the fresh sample is not always helpful, since the numbers present may be small (126). A more useful procedure is to hold samples at 7.2° C. (45° F.) for five days, then subject them to the S.P.C. at 32° C. (87). This gives any psychrophiles present an opportunity to grow, and counts will often be well into the millions. This is regarded as a much more satisfactory procedure than making a psychrophilic count on the fresh sample.

[1] This will probably be specified in the forthcoming 11th edition of *Standard Methods*.

Coliform bacteria. The presence of coliform bacteria in milk and its products does not have the same public health significance as does their presence in water supplies. Hence, in routine control work there is no point in attempting to differentiate between so-called fecal and nonfecal types.

For the routine examination of raw milk, interest in North America has been largely confined to certified milk (1), although one state (123) has a standard requiring freedom from coliforms in 1/100 ml. portions. It has generally been felt here that high counts represented growth more than direct contamination. With the widespread adoption of farm bulk tanks, growth will no longer be a factor, and more interest in the use of this test may be expected. The survey of milk supplies in eight U. S. cities reported in 1953 (38) showed some correlation between udder cleanliness and coliform content. The results of this survey (log. average coliform count 5,810 per milliliter) suggest there is room for considerable improvement.

In Britain, various authorities (62, 148, 165) have asserted that the coliform test gives the best indication of cleanliness of milk production; high coliform counts most frequently arise from neglected milking machines (148). The possibility that high counts may come from udders infected with coliform organisms must not be overlooked (164).

In pasteurized products, the use of the coliform test to detect recontamination has been more generally accepted. Stemming largely from the work of McCrady and his coworkers (109), the great value of this test, to both sanitarians and management, has steadily received wider recognition. The excuse that coliforms in pasteurized milk may not be heat-resistant was discredited by Buchbinder and Alff (27); the presence of coliforms in a pasteurized product definitely indicates recontamination and a potential health hazard. Pasteurized products, handled in clean, sanitized equipment, should be entirely free from coliform organisms. That this is possible is shown by the record of the Trifolium Dairy in Copenhagen (125); in 1955, 82.4% of samples were coliform-negative in 1-ml. portions after samples had been held at 17° C. (62.6° F.) for 24 hr. before testing. This procedure is employed by other European dairies. Encouraging results have also been reported by the Quebec Ministry of Health (7), where a limit of 50 coliforms per 100 ml. is in effect. The standard of not more than 10 per milliliter currently in vogue in the United States (168) is so lenient as to be of limited value; the presence of any coliforms should call for immediate investigation.

Two distinct types of test procedure, a plating technique and a tube dilution technique, are officially approved (2) in North America. Divergent opinions have been expressed concerning their relative merits. Where standards are stiff, as in Quebec (< 50 per 100 ml.), the dilution technique is preferred as larger volumes of milk can be tested (7). Where standards are more lenient (168), the plating technique is generally favored. Two shortcomings of the latter are (a) difficulty in deciding whether small red colonies should be counted, and (b) atypical colonies on overcrowded plates.

When applied to products containing other sugars in addition to lactose, e.g., ice cream, positive results must be confirmed to avoid misleading results.

False positive results have been reported where sweetened and unsweetened fresh fruits (bananas, peaches, strawberries) are added to the mix (12).

While 35° C. is currently the accepted incubation temperature on this continent (2), there is evidence that higher counts would be obtained with a lower incubation temperature. In Britain, the Coli-Aerogenes Sub-Committee of the Society of Applied Bacteriology, impressed with the evidence of numerous 37° negative strains, has recommended incubation at 30° C. (139). Belgian workers (174) report that over 50% of strains positive at 30° C. were negative at 35° C.

A unique procedure, the Bacto-strip method (55), has recently been developed for determining coliform counts. A definite volume of milk is absorbed by a sterile strip of filter paper impregnated with a suitable medium; the strip is returned to its plastic pouch and incubated 8–10 hr. The incubation period is inconvenient, and counts are generally lower than those by the regular plating technique (89). The tendency for colonies developing from motile strains to coalesce is a further drawback (60). Nevertheless, the method could be quite useful where no laboratory facilities exist.

The millipore filter technique has been advocated for the determination of coliforms in pasteurized and certified milk (48), but not enough information is available to date to judge of its value here. The use of a nonionic agent to facilitate filtration of 10 ml. of milk or 1 ml. of cream has been suggested (50).

Proteolytic bacteria. Organisms capable of proteolyzing casein are frequently responsible for undesirable flavors in dairy products. Surface taint of butter caused by *Pseudomonas putrefaciens* (43) is an example. The presence of most proteolytic organisms can be detected by plating on an agar medium containing 5–10% sterile skimmilk, and incubating at approximately 21° C. for three or more days. Colonies arising from proteolytic types will be surrounded by clear zones, where the protein has been digested by the enzymes they have secreted. A clear zone may also surround certain nonproteolytic bacteria which produce slight acidity; by flooding the plate with dilute acids, these zones around nonproteolytic colonies will disappear.

Lipolytic bacteria. Organisms attacking fats are often also proteolytic and also psychrophiles. This makes them particularly important in butter, cream, and cottage cheese, which are frequently held refrigerated for extended periods. The free fatty acids liberated from fats by hydrolysis contribute pungent flavor and odor to the product; these may be desirable in certain cheeses, but in other products they generally cause defects.

The enumeration of lipolytic bacteria is based upon detection of free fatty acids liberated from fat added to the culture medium; Nile blue sulfate is the indicator normally used. It stains normal fat globules pink; when the fat is hydrolyzed by lipolytic colonies, the globules stain blue. Care must be taken to avoid the toxic effect of an excessive amount of dye (96). One per cent of sterilized stained fat is added to the tempered medium just before pouring; the fat should be in the form of a fine emulsion. Tributyrin is frequently preferred to natural fat as it is more easily hydrolyzed; however, not all organisms hydrolyzing tributryrin also hydrolyze butterfat.

Molds and yeasts. Proper pasteurization of milk or cream destroys molds and yeasts; thus, their presence in a product indicates recontamination (22). Mold and yeast counts on butter are commonly run to check on plant sanitation; trouble with mold growth on butter is much less frequent when counts are low. The medium currently accepted here as standard (2) is potato dextrose agar, acidified to pH 3.5 before pouring to inhibit bacterial growth. Olsen and Bonner (124) recently reported 100 p.p.m. aureomycin even more useful for this purpose, yeast counts frequently being much higher than with the standard medium. Unpublished results from the writer's laboratory have confirmed this.

Mold and yeast counts are also employed by progressive cottage cheese manufacturers and sanitarians as indices of plant sanitation. A standard of not over 10 per milliliter is currently being met in some areas.

Bacto-strips (55) are also available for determining the mold and yeast count on equipment surfaces, in the air, etc. Good agreement with results from the conventional technique for determining air-borne contamination has been reported (89).

Mold mycelia count. When cream is stored on the farm at unsuitable temperatures for too long periods, growth of *Geotrichum candidum* takes place. Although the mold is killed by pasteurization, dead mycelia pass into the butter in appreciable amounts. They can be counted by a method (2) similar to that used to detect mold filaments in tomato products. This method can, therefore, tell much more about the lack of care the cream has been subjected to than can a viable count on the butter; it is frequently employed by food and drug officials and may be used as the basis for seizure and confiscation of butter.

Fermentation test. Milk for cheese-making is often tested by the methylene blue test, then incubation continued overnight in the fermentation test (153). It is believed by some that the type of curd formed indicates the types of bacteria present in the milk, particularly those causing gassy defects in cheese-making. However, the more commonly held view is that it is better to discriminate against milk heavily contaminated with bacteria; with lower-count levels the lactic acid bacteria in the starter have little difficulty in establishing dominance.

Keeping-quality tests. In Britain considerable emphasis is still placed upon keeping-quality tests for raw milk. Since almost all milk is pasteurized, and since there is usually little correlation between keeping quality before and after pasteurization, this interest seems rather archaic. Various methods have been tried out; of these, the clot-on-boiling (C.O.B.) test (45) is considered the most accurate, although it reflects chiefly souring. In this test, portions of the sample are maintained at 18° C.; morning and evening a fresh portion is immersed in boiling water and examined for clotting; this continues until clotting occurs. The method appears too cumbersome and time-consuming for routine testing.

As already indicated, the dye reduction tests are used to assess probable keeping quality of pasteurized milks in Europe. In the United States, TTC (26) and neotetrazolium (41) have been suggested for this purpose. In Europe, the coliform test after preliminary incubation is considered to be a more reliable

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indication of keeping quality (18, 126). In all of these procedures, the pasteurized sample is maintained at a temperature—usually 17–18° C. (62.6–64.4° F.)—favorable for the growth of saprophytic contaminants for a period before testing. The S.P.C. on pasteurized samples held at 7.2° C. for five days gives a good indication of the extent of recontamination, especially with psychrophiles responsible for spoilage during refrigerated storage.

Antibiotics. The presence of antibiotics in milk, either residual from therapy or by deliberate addition, can influence the results of bacteriological examinations (52, 92, 173), in addition to causing trouble in the manufacture of products dependent upon the lactic fermentation and the possible hazard to those individuals acutely sensitive to penicillin. Where the results of bacteriological tests are unexpectedly good, it may be wise to test for the presence of antibiotics.

Unfortunately, there is no rapid test whereby milk containing antibiotics can be detected and rejected. The incorporation of a suitable dye as a tracer has been reported on favorably (64, 149), but the method still lacks official sanction. This would appear to be the most satisfactory solution of this problem; producers would be much less prone to ship milk containing residual antibiotics if they knew that detection was simple. However, this would not preclude the possibility of the deliberate addition of antibiotics as preservatives.

A number of different tests have been reported for the detection of antibiotics in milk. All of these are based upon interference with bacterial growth and activity. One of the simplest is a starter activity test (146), patterned after that introduced by Horrall and Elliker (73); here, the extent of acid development when inoculated with a lactic starter and incubated for several hours is compared with that of a control. Care must be taken, however, to exclude the action of naturally occurring inhibitory substances. Greater sensitivity can be obtained by using *Streptococcus thermophilus* in place of the common starter streptococci (17). Another form of test utilizes a redox indicator to reflect interference with bacterial growth when incubated at a suitable temperature; triphenyl tetrazolium chloride is the indicator commonly recommended (120), although methylene blue (58, 143) and resazurin (115) have been used. The disk assay method has also been studied extensively. In its standard form (2), it is most useful for detecting the presence of penicillin; the test organism, *Bacillus subtilis*, is less sensitive to other antibiotics (88). An interesting modification of this method has recently been described (145), wherein the agar layer is flushed with resazurin; the completed test takes considerably less time than the standard disk assay method.

Hydrolytic rancidity. With the spread of bulk handling of milk there has been heightened interest in the occurrence of hydrolytic rancidity. A useful method for estimating the degree of its development has been developed by Minnesota workers (162).

ESTHETIC CONSIDERATIONS

Sediment. In addition to the microbiological tests already discussed, certain tests are commonly conducted which are concerned with esthetic considerations. Tests for sediment in milk (2) are usually applied to incoming raw milks. Dirt

has no place in milk; hence, its presence indicates carelessness in milking or handling. Milk containing in excess of 2.5 mg. per pint as determined by the off-the-bottom type of tester is customarily rejected. Unfortunately, a relatively clean disk may simply represent a good job of filtering on the farm, rather than care in production.

Different types of apparatus for sediment testing may give quite different results. Preliminary studies (80) have shown that the type operated by compressed air may collect appreciably less sediment on the disk than do certain other types.

There is also a tendency to consider that milk containing appreciable sediment is also high in bacteria. This is not necessarily so. Milk may contain appreciable amounts of sediment, yet show a reasonably low count; on the other hand, milk free from sediment may have picked up a heavy load of contamination from unclean equipment surfaces etc., or appreciable growth may have taken place.

Sediment tests are occasionally run on bottled milks. In most well-operated plants, however, the milk is subjected to filtration or clarification there in addition to straining on the farm, so the amount of sediment present is usually extremely small. Sediment tests for churning cream are also made by enforcement agencies; these require special facilities whereby the entire can of cream may be filtered (66). The visual mold test for cream (2, 129) might also be regarded as a form of sediment test. It is a useful additional criterion of churning-cream quality (122).

Milk for cheese-making may contain appreciable amounts of sediment, which is concentrated approximately elevenfold during the making process. The U. S. Food and Drug Administration has taken the lead in attacking this problem. In Canada since 1954, every vat of Cheddar cheese offered for grading by the federal grading service (111,884 in 1957) has been tested for extraneous matter, using essentially the procedure developed by Thibodeau (161). In 1957, federal grade standards (29) were amended to require a satisfactory extraneous matter test to qualify cheese for First Grade. This has greatly improved the picture, but it is suspected that it represents more effective dirt removal at farm and factory rather than cleaner milk production.

Decomposition. Tests for decomposition also may be regarded as concerned with esthetic considerations. Here tests for water-insoluble acids (WIA) and butyric acid (BA) in butter come to mind (71). Lipolysis in churning cream caused by growth of mold or lipolytic bacteria is reflected in higher values for these compounds in butter, and such evidence may be used by enforcement agencies to take legal action.

The souring of milk and cream also may be regarded as a form of decomposition. The titratable acidity test (2, 153) really measures the buffer capacity of the product. It is still widely used in the grading of churning cream; for market milk on this continent it has all but disappeared, but for manufacturing milk it is still used as a basis for rejection. Its great advantage is the speed with which it can be carried out. Care must be used when applying this test to milk

with high solids-not-fat; cases are known where even certified milk with counts under 10,000 per milliliter has been threatened with rejection because the titratable acidity was high (153). The effect of dilution of the milk or cream with water (2) in reducing the titratable acidity is not generally recognized (154).

The alcohol test (153), still used mainly to determine the suitability of milk for condensing, is primarily a reflection of acid development. Milk giving a positive reaction is usually within a few hours of curdling; on the other hand, some milks quite satisfactory for pasteurization may react positively (32).

Lactic acid is the chief product of bacterial growth in most milk samples; a rapid test for its detection and measurement would be most helpful. Based on the method of Ling (102), Clegg and coworkers (133, 158) have described a rejection test which can be completed in 5 min.; a value of 0.03% is recommended as the lowest value of apparent lactic acid at which milk could be rejected. However, even a 5-min. delay on the receiving platform would disrupt operations in many plants. A test of this type might replace the titratable acidity test in the examination of nonfat milk solids (29); at present careful neutralization makes possible the use of substandard raw material without detection.

Mastitis. While mastitis is primarily a problem of animal health, and occasionally is a factor in the transmission of human pathogenic organisms, the inclusion in the milk supply of the secretion from infected udders also may be regarded as an esthetic problem. Regardless of the use to which it is to be put, no milk can be considered to be of satisfactory quality if drawn from diseased udders. Milk is customarily defined, in part, as the product of the uninterrupted milking of one or more healthy cows, yet the average leucocyte count on samples in the 1953 U. S. survey (38) was 680,000 per milliliter. McKenzie (110) has recently reported that where the leucocyte count exceeded 100,000 per milliliter, the solids-not-fat content was correspondingly reduced. Certainly, a count on herd milk in excess of 500,000 per milliliter strongly suggests an appreciable number of infected udders in the herd.

Various procedures have been suggested for the detection of milks carrying excessive numbers of leucocytes. The direct microscopic examination of smears (2), using a low-powered objective, can yield very useful information in the hands of a trained worker. The ability of resazurin to reflect the weak reducing activity of leucocytes (137) can be used to indicate those herds in need of examination; in either the triple reading test (2, 91) or Hempler's pink test (68, 69), rapid, early color change, followed by a lag for an hour or more, should be regarded with suspicion. Tests can be carried out at the farm, also, preferably using Golding's dried vial technique (108).

A simple rapid procedure, which is rapidly gaining in popularity, is the Whiteside test (117, 171). It is effective in showing differences in herd milks, and can be used as a platform test; it can also be used by the fieldman, or by the farmer himself, to check individual cows or quarters. It has considerable educational value, since the farmer can readily observe how milk from normal and from infected animals differs in appearance. Favorable reports have appeared from various countries; one from Sweden (3) states that the Whiteside test was

more valuable than the catalase or the toxin–blood agar tests, and recommends its adoption. The California Mastitis Test (CMT) (142), developed from the Whiteside test, is designed primarily for checking individual quarter samples; it is much more sensitive than the strip-cup test, and is the basis of a promising mastitis control program (72).

The tests mentioned so far are indirect tests, useful for directing attention to certain milk supplies. A search for specific causative organisms also can be made; information from such tests is a valuable guide in treatment of mastitis. Samples should be taken aseptically from individual quarters. The simplest procedure is probably the Hotis test (74), especially good for detecting *Streptococcus agalactiae*. Use of blood agar, either as streak or pour plates, is considered the most reliable method; the type of colony and hemolysis aids in differentiating various common mastitis organisms. The CAMP test (31) is very useful in identifying *S. agalactiae* by a typical hemolytic reaction. Fuller information on various aspects of mastitis, including testing, is given in Plastridge's review (134).

HEALTHFULNESS

Over the years, milk and its products have been involved in the spread of a number of diseases of human as well as of bovine origin (2). In North America and some European countries, marked progress in the elimination of bovine tuberculosis and brucellosis has sharply reduced the hazard to those still consuming raw milk, while the pasteurization of all but a small fraction of the milk supply has reduced the amount of milk-borne disease to insignificant proportions (39, 150).

Nowadays, routine laboratory testing of raw milk supplies in regard to safety is largely confined to the milk ring test (2) as a screening test to detect brucellosis. This simple, inexpensive test is so sensitive that when applied to pooled herd samples a single infected animal in a herd of 40 can be detected (79). Like all biological tests, it has its limitations. False positive tests occasionally occur in milk from noninfected cows, especially fresh cows or those suffering from mastitis; infected nonlactating animals escape detection, whereas milk from a small percentage of infected animals gives a negative reaction. Despite these limitations, the test has been of inestimable value in programs for the elimination of brucellosis (79).

Phosphatase test. Although it was accepted that efficient pasteurization made milk safe, health officials had no assurance that the procedure had been properly carried out. With the development of the phosphatase test by Kay and Graham (95), a routine analytical method first became available that could give fairly substantial assurance. Underpasteurization, or recontamination with as little as 0.1–0.2% of raw milk or cream will result in a positive reaction. It is still possible for small amounts of underpasteurized milk to be mixed with fully pasteurized milk and escape detection, but judging by the greatly diminished number of outbreaks of milk-borne disease (39, 150), the public health hazard is slight.

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Various modifications of the original phosphatase test have been developed; two of these, the New York State Department of Health and the Sanders and Sager methods, are accepted here as official methods (2), whereas the Scharer method is only a screening procedure. A modification developed by Aschaffenburg and Mullen (9) is simple and rapid enough for a routine plant test, yet compares in sensitivity with the Kay-Graham test (95). Directions must be followed carefully, to avoid false positive reactions. Flavors and coloring compounds in ice cream which possess phenolic rings may give false positive reactions. False positive reactions also may result from growth of certain species of microorganisms which produce phosphatase (13, 63). Means of differentiating microbial phosphatases from milk phosphates have been described (98). More recently, reactivation of phosphatase in milk or cream processed at higher temperatures (176) has caused some concern to control officials. More emphasis upon keeping samples cold until tested would go a long way toward the solution of this problem, although an intensive study of reactivation is undoubtedly needed.

Cheese made from raw milk, especially Cheddar and its modifications, has been responsible for numerous outbreaks of enteric disease. Regulations usually require that Cheddar cheese sold when less than 60 days old be made from pasteurized milk. The Sanders and Sager method (141) is a modification of the phosphatase test expressly designed to overcome the strong buffering capacity of cheese and the presence of interfering substances. That of Kosikowski (97) is another. Phosphatase tests for cheese are fully discussed in a recent F.A.O. monograph (98).

While the phosphatase test may be applied to freshly churned butter to determine whether or not it was made from pasteurized cream, unsatisfactory results have attended its application to storage butters (95, 128).

Recently, interest has been aroused by food-poisoning outbreaks attributed to the presence of staphylococcus enterotoxin in nonfat milk solids (4, 8) and in Cheddar cheese and its modifications (6, 159, 160). While toxin production in raw milk itself rarely presents a hazard to health (93), due to the repression of growth of staphylococci by other types, some growth may take place both before and during the cheese-making process (107, 157). Cheese with excessive numbers of coagulase-positive staphylococci must, therefore, be regarded with suspicion (160). Counts of staphylococci in cheese are much higher during the warmer months (157); greater emphasis on prompt cooling of night's milk should reduce the hazard here.

Reliance can not be placed entirely upon low plate counts, however. In the manufacture of powder or in subsequent storage, the staphylococci may be killed off or die, but the enterotoxin remain. This was the case in the Puerto Rico outbreaks (8). Faulty plant practice, rather than excessive growth of staphylococci in milk before it reaches the plant, is more likely to be responsible for the presence of toxin in the powder (4).

GENERAL OBSERVATIONS

There is an unfortunate tendency for many people to forget that bacteriological determinations on milk or milk products are far from being precise

measurements. Making fine distinctions between samples on the basis of small differences in plate counts, for example, is entirely unwarranted, and is expressly discouraged in *Standard Methods* (2). It should further be emphasized that conclusions as to the quality of a milk supply should be drawn only after examining a series of samples, not on the basis of a single sample. This is recognized in the Standard Milk Ordinance (168), where either the logarithmic average of the last four samples is taken or else one high count out of four is not penalized.

Because milks may show appreciable differences in bacteriological content from day to day, it is desirable that testing be carried out as frequently as possible. The less expensive dye reduction tests have an advantage here; they also indicate substandard supplies with a minimum of delay, so that the fieldman can check at once for the source of the trouble.

It should be emphasized once again that the hygienic quality of milk embraces a number of factors. No one method can furnish all the information required in quality control. In addition to estimating the total bacterial population, tests for thermoduric organisms and for excessive leucocyte counts are essential to the control of raw milk supplies; for pasteurized milk the coliform test, or plating after refrigerated storage for several days, is more valuable than the S.P.C. in indicating the care taken to minimize recontamination.

Probably because America has been in the van of progress in respect to milk hygiene, there has been a tendency to overlook testing methods developed elsewhere. This attitude appears to be changing. It seems probable that such developments as the Aschaffenburg and Mullen (9) modification of the phosphatase test, the use of penicillin in media for coliform testing (104, 147), the roll-tube procedure (5), and the coliform test on pasteurized products after previous incubation (16, 18, 125) could be used here to advantage.

The widespread adoption of farm bulk tanks makes it necessary to reappraise standards and methods of testing developed for milk in cans. Some means of encouraging the growth of contaminants prior to testing appears necessary, if bacteriological analysis is to give a reliable indication of the care taken in producing and handling milk. Such a procedure, applied to all milks regardless of the method of handling, appears less open to objection than that of setting up two different standards for acceptable milk (36). Furthermore, the adoption of preincubation would make possible a marked improvement in many milk supplies without altering currently accepted standards for milk handled in cans. In effect, it would mean acceptance of the principle on which control of raw milk has long been based in Britain—that testing freshly taken samples, particularly if milks have been well-cooled, may yield misleading results.

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CHARACTERISTICS OF HIGH-MELTING TRIGLYCERIDE FRACTIONS FROM THE FAT-GLOBULE MEMBRANE AND BUTTER OIL OF BOVINE MILK[1]

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SUMMARY

A high-melting triglyceride fraction (HMGF) was isolated from an alcohol-ether extract of the fat-globule membrane and from an ethanol extract of butter oil. The HMGF was characterized by its iodine value (4.8-5.0), saponification equivalent (200-202), and melting range (50.0-52.5° C.), and by its ultraviolet (220-340 mμ), near-infrared (1.6-3.1 μ), and infrared (2-14 μ) spectra. The fatty acid composition of the fraction was determined by converting the glyceride to its constituent fatty acid methyl esters which were resolved by gas chromatography as follows: C_{10} — 0.1, C_{12} — 0.9, C_{14} — 11.0, C_{16} — 59.6, C_{18} — 16.5, $C_{18}^{1=}$ — 5.6, $C_{18}^{2=}$ — 0.4, $C_{18}^{3=}$ — 0.2, and unassigned components—5.8 mole %, respectively. Characteristics of the high-melting glyceride fractions obtained from the membrane and from butter oil were similar. The HMGF constitutes the triglyceride content of the membrane.

In 1933, Palmer and Wiese (6) reported the isolation of a high-melting triglyceride from the fat-globule membrane prepared from washed cream. Later, Jenness and Palmer (4) published specific chemical properties of the ethanol-insoluble fraction extracted at 24–25° C. from buttermilk, butter serum, and butter oil. A recent note by Patton and Keeney (7) gave characteristics of a glyceride isolated from an acetone extract of milk fat. This paper presents the results of a study conducted independently from but concurrently with the recent work of Patton and Keeney. The results presented here are based on a high-melting triglyceride fraction (HMGF) isolated from the fat-globule membrane and a similar fraction obtained from butter oil as ethanol-insoluble fractions at 22° C.

METHODS

The iodine values, saponification equivalents, and melting-range values were determined according to standard procedures (1).

Ultraviolet and near-infrared spectra of the HMG Fractions and other selected materials were obtained with a Beckman Model DK-2 ratio-recording spectrophotometer.

A Beckman Model IR-5 double-beam infrared spectrophotometer was employed to study the spectral range from 2 to 14 μ. Samples were carried in spectrophotometric-grade carbon tetrachloride.

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[2] This article is part of a dissertation to be presented to the Faculty of the Graduate School of Michigan State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The HMG Fractions and butter oil were prepared for gas chromatographic analyses by methanolysis in Skellysolve A, according to the method described by Smith and Jack (9). Excess solvent was removed in vacuo. The resulting fatty-acid methyl-ester mixtures were resolved on a Burrell gas chromatographic unit. Identification of the unknown chromatographic peaks was achieved by comparing their elution characteristics with those from similarly prepared chromatograms of a known fatty acid mixture, and by employing the known mixture as an internal standard. The chromatographic column was packed with 18.6 g. of 20% (w/w) Reoplex 400 on 60–80 mesh Celite 545 and was operated at a temperature of 224° C. The detector was maintained at 238° C. while the preheater was operated at 263° C. Helium was employed as the carrier gas, with an outflow rate of approximately 50 cc/38.9 sec. at C_{18} for all chromatographic runs.

EXPERIMENTAL PROCEDURE

Preparation of the high-melting triglyceride. The glyceride fraction was obtained from the concentrated fat-globule membrane prepared from washed cream (3). The membrane material was washed several times with peroxide-free ethyl ether at approximately 20° C., to remove fat which was not associated with the membrane complex. Lipids complexed within the membrane were extracted with a 35% ethanol in ethyl ether mixture for 15 min. at −10° C., followed by filtration and two washings with cold ether to remove the excess ethanol. Five additional extractions with ether at 35° C. were employed to remove the last traces of lipids from the membrane proteins. These alcohol and ether extracts were combined and evaporated to dryness in vacuo at 40° C. The residual lipids were taken up in five volumes of acetone. The mixture was allowed to stand for 30 min., after which the phospholipid precipitate was filtered off and the clear filtrate evaporated to dryness in vacuo. At this point, it should be realized that acetone does not remove quantitatively the heterogeneous phospholipid mixtures (5). The remaining phospholipids were not recovered in the HMG Fractions.

The crude glyceride was dissolved in 600 ml. of absolute ethanol at 35° C. and allowed to stand overnight at 21–23° C. to allow crystallization of the HMGF. The copious white precipitate was separated from the yellow supernatant by vacuum filtration. This crystallized fraction was purified further by four crystallizations from absolute ethanol at 21–23° C. The purified glyceride fraction was freed of ethanol in vacuo and stored under nitrogen until analyzed. Similarly, 100 g. of anhydrous butteroil was extracted with 1,500 ml. of ethanol at 35° C. and the HMGF allowed to crystallize at 21–23° C. The precipitate was treated similarly to the membrane HMGF and represented the HMGF of butter oil.

Following each crystallization of the membrane HMGF, a sample of the ethanol supernatant was analyzed in the ultraviolet region, to determine the extent of removal of ethanol-soluble materials which, if present, would serve to mask the true characteristics of the HMGF. These spectra are shown in Figure 1. The characteristics of the spectra differ markedly as the number of crystallization steps is increased. Following the third extraction, the spectrum

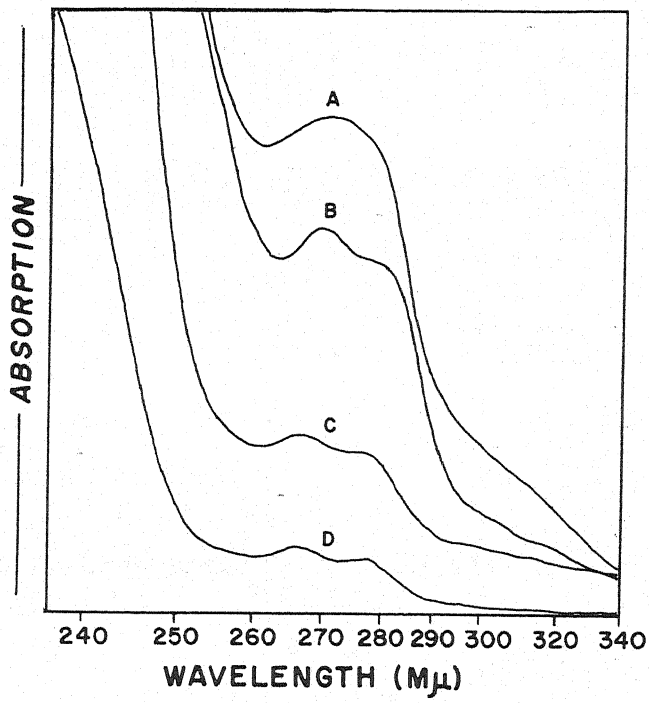


FIG. 1. Ultraviolet spectra of ethanol extracts of the high-melting triglyceride. Curve A, first extract, diluted 1: 2, 0.1 cm. path. Curve B, second extract, diluted 1: 1, 0.1 cm. path. Curve C, third extract, undiluted, 0.1 cm. path. Curve D, fourth extract, undiluted, 1.0 cm. path.

of the ethanol supernatant began to resemble that of the HMGF (Figure 2, B). The first and second extractions, which were yellow in color, showed spectra quite dissimilar to those of the subsequent extractions. Obviously, this was due to the presence of absorbing substances such the sterols, beta carotene, or phospholipids. These data indicate, then, that four extractions with absolute ethanol were required to free the membrane HMGF of its major impurities. The ethanol extractions were combined, evaporated to dryness in vacuo, and retained for further study.

Characterization of the high-melting triglycerides. The iodine value, saponification equivalent, and melting range for the HMG Fractions of this study were compared with similar values for the glyceride fractions reported by Jenness and Palmer (4) and by Patton and Keeney (7), Table 1. A difference exists between the reported iodine values of the two HMG Fractions reported here and the HMGF obtained by acetone fractionation from milk fat (7). This difference might be attributed to the method of preparation; that is, ethanol extraction as opposed to acetone extraction.

The ultraviolet spectra of the HMG Fractions from the fat-membrane and butter oil are compared in Figure 2. These glycerides show identical spectral characteristics with five absorption maxima at 315, 301, 279, 267, and 256 mμ, respectively. Smith and Jack (9), reporting on the unsaturated fatty acids of

TABLE 1

Properties of the HMGF isolated from the fat-globule membrane and from milk fat

Analysis	From the fat membrane (this study)	From milk fat (this study)	From the fat membrane (4)	From milk fat (7)
Iodine value	5.0	4.8–4.9	5.0–7.1	2.0
Saponification equivalent	201–202	200–202	198.8–204	....[c]
Melting range	50–51.5° C.	50–52.5° C.	52–53° C.	....[c]
Refractive index at 60° C.	1.4455	1.4453	.....	....[c]
Yield	44.48%[a]	4.88%[b]		

[a] Percentage of bound lipid in membrane.
[b] Percentage of original butter oil.
[c] Reported by Keeney and Patton (7) to be similar to values reported by Jenness and Palmer (4).

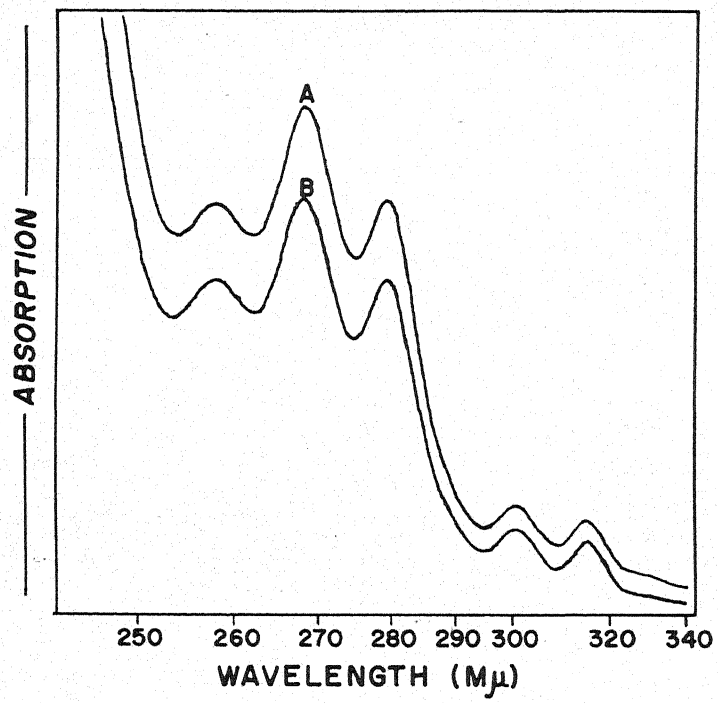
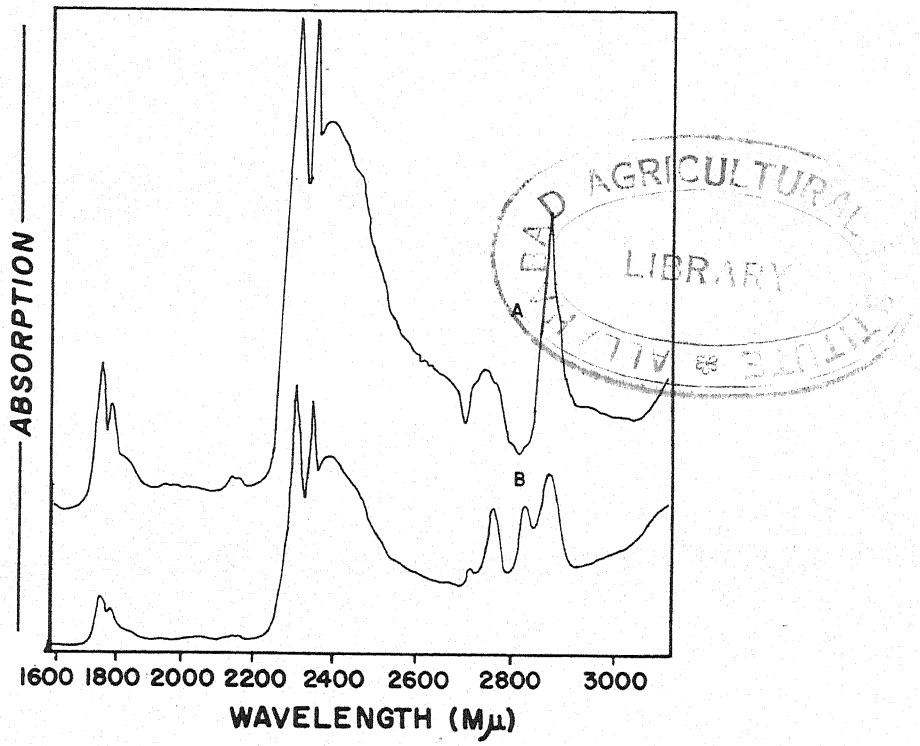


FIG. 2. Ultraviolet absorption spectra of the high-melting glyceride fractions obtained from (A) butter oil and (B) the fat-globule membrane.

milk fat, present almost identical spectral characteristics, showing absorption peaks at approximately 313, 300, 280, 268, and 233 mμ, but not at 256 mμ. These spectral qualities characterize the presence of unsaturation in the glycerides. However, the data can not be interpreted to determine the type of conjugation, since alkali isomerization was not employed.

In recent years, the near-infrared region of the spectrum (2.7–3.0 μ) has proven useful for the study of functional groups and has been especially useful in the identification of hydroperoxides (8). Figure 3 shows the near-infrared



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FIG. 3. Near-infrared (photoelectric) absorption spectra of (A) tristearin and (B) the high-melting triglyceride in CCl_4. Concentrations approximate 5%.

spectra for the membrane HMGF, and tristearin. An examination of these spectra reveals that there was little similarity between these compounds in the region of 2.7–3.0 μ. Obviously, this dissimilarity arises from the nature of the molecule, i.e., saturation or unsaturation.

The infrared spectra (2–14 μ) of the HMG Fractions and tristearin are shown in Figure 4. Although this spectral region is most useful in assigning functional groups, one must be cautioned against over-simplification when interpretations are based on these data alone. In the case of lipids, for example, the spectra of the fatty acids correspond closely to related spectra of triglycerides. One would expect this correlation because of the similarities in the functional groups of the related compounds. The esters, on the other hand, differ in their spectral characteristics, depending on the type of ester (i.e., methyl or ethyl). The spectra of tristearin and of the HMG Fractions were quite similar and corresponded to those published by Patton and Keeney (7).

Gas chromatograms of the methyl esters of the fatty acids of butter oil and the membrane HMGF from the same milk, and a mixture of known fatty acids (C_{10} to C_{18}, and $C_{18}^{=1}$ to $C_{18}^{3=}$) are shown in Figure 5. These chromatograms were analyzed and compared with similar data reported by Patton and Keeney (7), Table 2. The membrane HMGF contained approximately 9 *M* % more C_{16} and 9 *M* % less C_{18} saturated acids, and 2.0 *M* % more C_{18} unsaturated acids

TABLE 1

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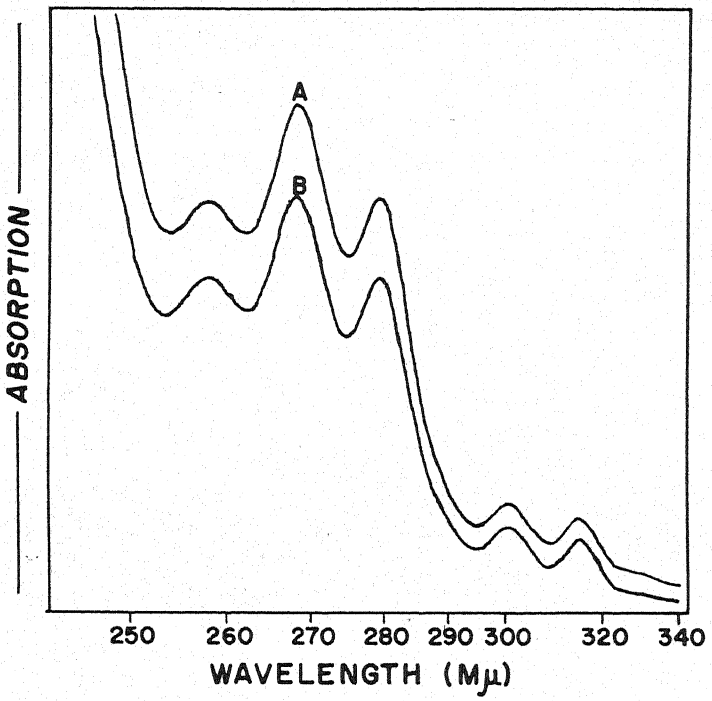
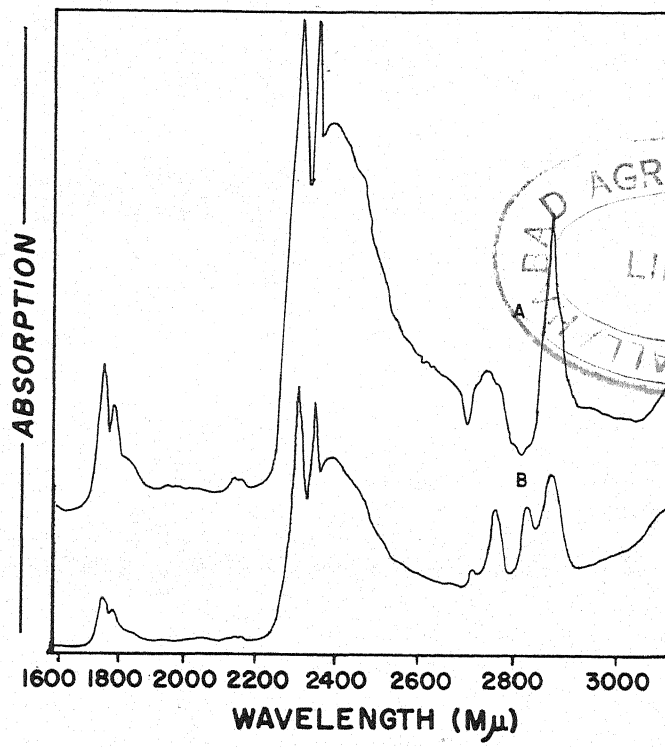


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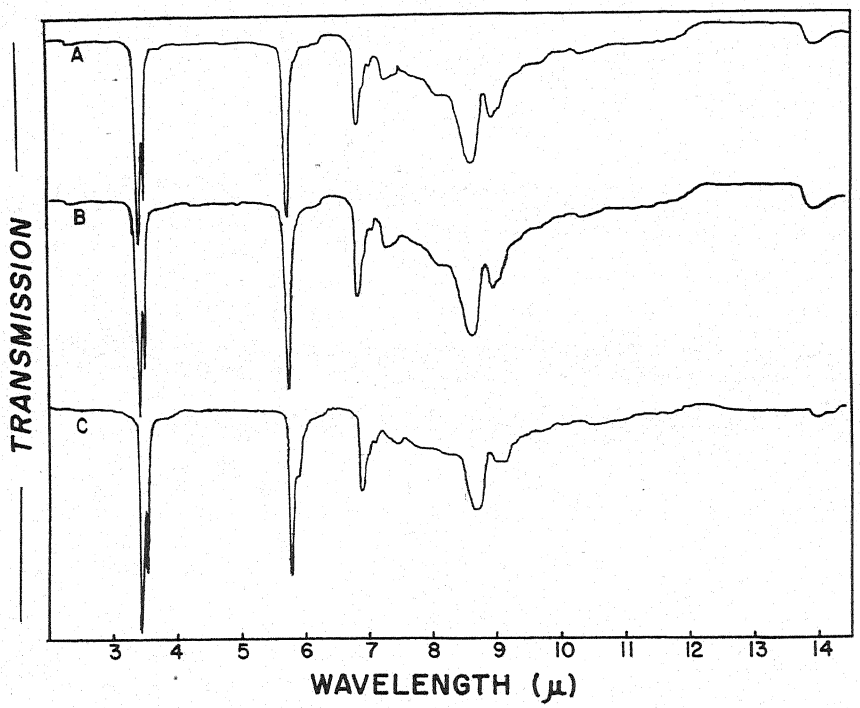


FIG. 4. Infrared spectra of (A) HMGF from butter oil, (B) HMGF from the fat-globule membrane, and (C) tristearin. Concentration was approximately 5%.

TABLE 2

A comparison of the fatty acid composition of the fat-globule membrane HMFG, butter oil from the same preparation, and a HMFG isolated from milk fat

Fatty acid	HMGF from the fat membrane[a]	Butter oil	HMGF from milk fat (7)	
	(*M* %)			
			A–1	B–1
C_{10}	0.1	3.9	0.3	0.7
C_{12}	0.9	4.3	1.4	1.6
C_{14}	11.0	12.6	12.1	13.6
C_{16}	59.6	25.5	50.5	51.6
C_{18} saturated	16.5	11.3	25.5	30.4[c]
C_{18} unsaturated	6.1	28.3	4.1[b]	.....
$C_{18}^{1=}$	5.6	25.8		
$C_{18}^{2=}$	0.4	.3		
$C_{18}^{3=}$	0.2	2.2		
Unassigned peak areas	5.8	10.5	5.9	.....

[a] Identical gas chromatograms were obtained for both the membrane and butter oil HMG Fractions.
[b] Total C_{18} unsaturated.
[c] Includes C_{18} unsaturated.

than the glyceride reported by them. Specifically, the C_{18} unsaturated acids have been designated as $C_{18}^{1=}$—5.6 *M* %, $C_{18}^{2=}$—0.4 *M* %, and $C_{18}^{3=}$—0.2 *M* %. No analysis was made for $C_{18}^{4=}$. Unassigned peaks accounted for 5.8 *M* % of the total mixture. The data for the butter oil sample were presented merely for the purpose of establishing the nature of original milk fat.

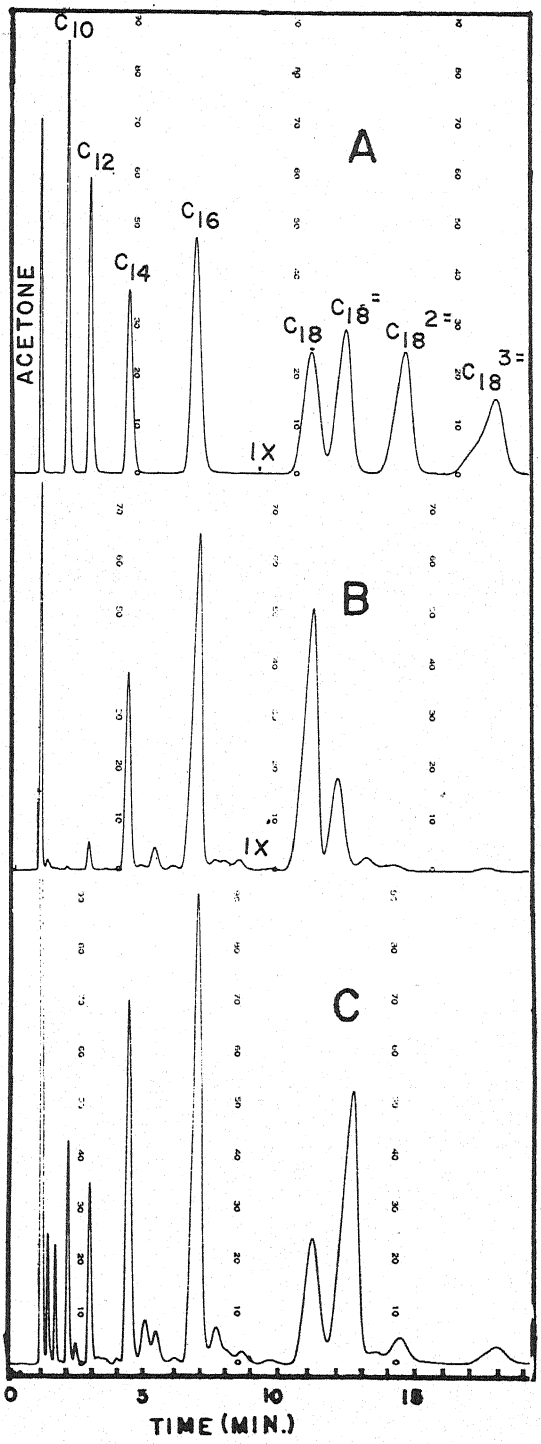


FIG. 5. Gas chromatograms of the fatty acid methyl esters of (A) a known mixture of fatty acids, (B) the fat-globule membrane HMGF, and (C) butter oil. 1x denotes time of a twofold increase in detector sensitivity.

Interestingly, the chromatogram of the esterified ethanol-soluble fraction of the membrane showed only traces of fatty acids, indicating a complete recovery of the HMGF from the ethanol crystallization step, as well as suggesting that the entire membrane triglyceride is HMGF. A classification of the unsaponifiable materials (beta carotene, sterols, etc.), constituting the major portion of this fraction, is currently in progress in this laboratory.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Edward M. Priest, M.D., and his associates in the Division of Cardiology at the Henry Ford Hospital, Detroit, for performing the gas chromatographic analyses of the methylated fatty acid samples reported in this paper.

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COMPARISON OF NUCLEIC ACIDS AND OTHER PHOSPHORUS COMPOUNDS OF TERRAMYCIN-SENSITIVE AND -RESISTANT STRAINS OF *STREPTOCOCCUS LACTIS*[1]

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SUMMARY

Terramycin-sensitive and terramycin-resistant strains of *Streptococcus lactis* 10A were inoculated in an enriched broth and incubated at 30° C. After 6, 12, and 24 hr. of incubation, determinations were made for total cellular matter, acid-soluble phosphorus, lipid phosphorus, nucleic acid phosphorus, protein phosphorus, and ribonucleic and deoxyribonucleic acids.

The sensitive strain consistently produced more cellular matter than did the resistant strain. The concentrations of the various phosphorus fractions fluctuated during incubation. In general, acid-soluble phosphorus was present in the highest concentrations in cells of both the strains, followed by protein phosphorus, lipid phosphorus, and nucleic acid phosphorus, in that order. At all times, cells of the sensitive strain contained higher concentrations of various phosphorus fractions, except for the acid-soluble fraction, which was present in higher concentration in the resistant cells after 6 and 12 hr. of incubation, and except for the lipid phosphorus content, which was higher in the resistant cells after a 12-hr. incubation period. The ribonucleic acid contents of both cultures were three to four times higher than their deoxyribonucleic acid contents. The total nucleic acid content of the sensitive cells during the earlier stages of incubation was appreciably greater than that of the resistant cells, indicating that the sensitive culture synthesized the nucleic acids more rapidly. However, the total nucleic acid content of the sensitive cells varied widely in different trials. The total nucleic acid content in both the strains decreased after 12 and 24 hr. of incubation, but the extent of decrease in the sensitive strain was more rapid than in the resistant strain, with the result that after 24 hr. of incubation, the total nucleic acid content of both the strains was approximately the same.

With the thought that antibiotic-resistant cultures may lend themselves to use in the manufacture of cultured products from antibiotic-milk, four strains of *Streptococcus lactis* were made resistant to 100 p.p.m. of terramycin (1, 15). Physiological studies on terramycin-sensitive and terramycin-resistant strains of *S. lactis* 10A, reported elsewhere (1, 14, 15, 16), have revealed considerable physiological differences between the two strains with respect to their cell number multiplication, acid production, utilization of phosphate esters and radioactive phosphorus, and metabolism of certain carbohydrates. In general, it was observed that the resistant strain metabolized at a reduced rate compared to the sensitive strain.

Nucleic acids combined with protein constitute an essential part of all animal and plant cell-nuclei which play an important part in the physiological functioning of the cells. Because of the physiological differences between the sensitive

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and resistant strains of *S. lactis* 10A observed in previous studies, it was felt that differences might exist in their nucleic acid and nucleoprotein metabolism. These studies were conducted to investigate whether there existed any differences between the nucleic acid, nucleoprotein, and other phosphorus-containing fractions of the terramycin-sensitive and -resistant strains of *S. lactis* 10A.

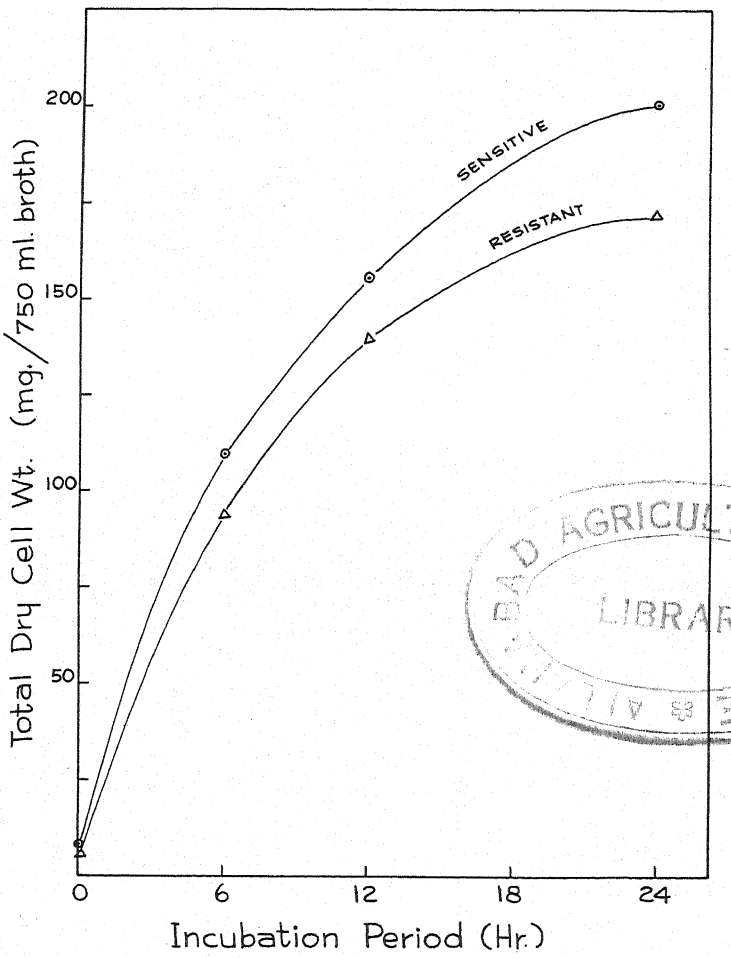
EXPERIMENTAL PROCEDURE

The terramycin-sensitive and -resistant strains of *S. lactis* 10A were grown in an enriched broth (pH 6.8) consisting of 0.5 g. yeast extract, 0.5 g. lactose, 0.5 g. dibasic potassium phosphate, 0.25 g. neopeptone, and 0.25 g. peptonized milk per 1,000 ml. Freshly prepared medium was inoculated with 5% of a 12-hr. broth culture and incubated at 30° C. At the end of 6, 12, and 24 hr. of incubation, the cells were collected by centrifugation, washed two or three times with sterile water, and suspended in 10 ml. of sterile water. The total dry cellular material produced at the end of each time-interval was determined on a 2- to 5-ml. aliquot of the suspension by means of the Mojonnier technique (9). Another aliquot was used for the determination of various phosphorus fractions and nucleic acids by employing the step-wise fractionation procedure of Schneider (11, 12). The cell-suspension was mixed with a small quantity of sand and ground for 10 min. with a pestle and mortar to facilitate extraction of various phosphorus fractions. The phosphorus fractions determined were acid-soluble phosphorus, lipid phosphorus, nucleic acid phosphorus, and phosphoprotein phosphorus. The acid-soluble phosphorus fraction was extracted with 10% cold trichloroacetic acid and separated from the residue by centrifugation. The lipid phosphorus fraction was extracted from the residue with alcohol and hot alcohol: ether treatment. After the removal of the second fraction, the nucleic acid phosphorus fraction was extracted by boiling the residue with 5% trichloroacetic acid. Finally, the protein-attached phosphorus was determined after alkali hydrolysis of the residue. In all of the fractions, phosphorus was determined by the standard Fiske and Subbarow Method (7). Ribonucleic acid (RNA) was determined by the orcinol-pentose colorimetric method of Brown (4), as described by Schneider (12). Since orcinol reacts with both ribonucleic acid and deoxyribonucleic acid (12), a correction was applied for the presence of deoxyribonucleic acid. Deoxyribonucleic acid (DNA) was determined by the diphenylamine reaction of Dische and Schwarz (6), as modified by Schneider (12). The procedures for the nucleic acids have been described fully by Volkin and Cohn (17). Commercially available ribonucleic acid and deoxyribonucleic acid [3] were used for preparation of the standard curves for nucleic acids. All results presented in this study are expressed on a dry weight basis.

RESULTS AND DISCUSSION

The dry cellular matter produced by the sensitive and resistant strains of *S. lactis* 10A is illustrated by Figure 1. Dry cellular weight at zero-hour incu-

[3] Obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.



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FIG. 1. Total dry cellular matter produced by terramycin-sensitive and -resistant *S. lactis* 10A. These data represent average values of three to four trials conducted with each culture at each time of incubation.

bation represents the estimated values calculated from the amount of the inoculum. The resistant strain produced 10 to 14% less cellular material than did the sensitive strain. In a previous study (15), the rate of multiplication of the two strains was found to be the same, as determined by the viable cell total count. In the present work, the growth was determined by the total dry cellular matter produced by the organisms at various time-intervals of incubation. The variations in the two studies might be due either (a) to the production of longer streptococcal chains by the sensitive strain which may be more resistant to being shaken apart during dilution, (b) to the production of smaller-sized cells by the resistant strain, or (c) to the fact that the sensitive strain multiplied faster and approached the decline phase earlier than the other strain and, thereby, the dead cells of the sensitive strain contributed to the higher cellular weight.

The data for the various phosphorus fractions are presented (Table 1). At the end of 6 and 12 hr. of incubation, the resistant cells usually contained a higher concentration of acid-soluble phosphorus but a lower concentration of lipid, nucleic acid, and protein-bound phosphorus than the sensitive cells. However, at the end of 24 hr. of incubation, all of the phosphorus fractions were present in lower concentrations in the resistant cells than in the sensitive ones. In general, the acid-soluble phosphorus was present in the highest concentration in cells of both strains, followed in order by the protein phosphorus, lipid phosphorus, and nucleic acid phosphorus.

TABLE 1

Phosphorus content of the terramycin-sensitive and -resistant *Streptococcus lactis* 10A[a]

Phosphorus fraction	*S. lactis* 10A	Phosphorus content mg/g		
		6 hr.	12 hr.	24 hr.
Acid soluble-P	Sensitive	3.66	4.54	5.58
	Resistant	4.22	6.20	3.11
Lipid-P	Sensitive	2.15	1.72	2.12
	Resistant	1.69	1.80	1.94
Nucleic Acid-P	Sensitive	1.56	1.28	1.47
	Resistant	1.25	0.95	1.19
Protein-P	Sensitive	2.92	3.48	2.08
	Resistant	2.57	3.11	1.91

[a] These data represent the average results of from three to five trials.

Results of nucleic acid analyses are presented in Table 2. The average nucleic acid content in the resistant cells was lower than that in sensitive organisms, at all incubation intervals. Wide variations existed in the nucleic acids content of the two strains in different trials. At the end of 6 hr., the resistant cells contained 31.9 to 40.3 mg/g (average 35.4 mg/g) of the nucleic acids, compared to 38.3 to 56.1 mg/g (average 50.0 mg/g) by the sensitive cells. At 12 and 24 hr. of incubation, the cells of both the strains contained a lower concentration of

TABLE 2

Nucleic acid content of the terramycin-sensitive and -resistant *Streptococcus lactis* 10A at the end of various incubation periods

Trial No.	Compound	Sensitive			Resistant		
		6 hr.	12 hr.	24 hr.	6 hr.	12 hr.	24 hr.
		(mg/g)					
1	Ribonucleic Acid	50.9	18.0	25.5	29.7	16.8	21.6
2		28.6	26.4	20.6	21.1	22.8	19.3
3		45.4	18.8	34.7	33.1	27.2	30.7
4		.....	33.9	.....	.....	24.1	.....
Average		41.6	24.3	26.9	27.9	22.7	23.9
1	Deoxyribonucleic Acid	5.2	6.8	9.8	4.5	2.9	12.6
2		9.7	10.7	7.0	10.8	7.7	7.9
3		10.3	8.5	9.4	7.2	6.1	8.8
4		.....	10.8	.....	.....	5.8	.....
Average		8.4	9.2	8.7	7.5	5.6	9.8
Average total nucleic acid		50.0	33.5	35.6	35.4	28.3	33.7

these acids than they did at 6 hr. In both strains and at all times, ribonucleic acid was more predominant than deoxyribonucleic acid, and constituted 72 to 83% of the total nucleic acid content in the sensitive cells and 71 to 80% of the total nucleic acid content in the resistant cells.

The finding of higher concentrations of ribonucleic acid in the young cells than in the older cells seems to corroborate the report of Morse and Carter (10), who observed that *Escherichia coli* synthesized a large amount of ribonucleic acid during the lag period. Also, Belozersky (2) has shown that the nucleic acid of bacteria, yeast, and mold varied between 3 and 29%, depending upon their age.

Comparison was made of two methods of determining nucleic acid–phosphorus—one involving the actual determination of phosphorus and the other involving calculation of phosphorus from the nucleic acids found (Table 3). It was observed that nucleic acid–phosphorus actually determined (Column 5) was always lower than the nucleic acid–phosphorus calculated from the nucleic acids found (Column 4). Schneider (12) observed that although all of the phosphorus in the trichloroacetic acid extract of animal tissues could be accounted for by nucleic acids, the nitrogen found was always higher than that calculated from the nucleic acids present. He suggested that this could mean either that the nucleic acids present did not conform to the accepted tetranucleotide structure or that some other nitrogenous compound was being extracted by the acid treatment. In the present study, the disagreement between the nucleic acid–phosphorus actually found, and that calculated, may suggest either that in the process of extracting the nucleic acid fraction all of the terminal phosphorus groups can not be extracted or that the phosphorus contents of the nucleic acids in the sensitive and resistant *S. lactis* are less than the accepted phosphorus content of nucleic acids.

Sevag *et al.* (13) have shown that in 16-hr.-old cells of *Streptococcus pyogenes* total nucleic acids (determined on the basis of purine nitrogen) constituted 14.8 to 24.1% of the dry weight of the cells. In the present study, the nucleic acids content (determined on the basis of their sugar content) of the sensitive and resistant strains of *S. lactis* was found to vary from 3.3 to 5.0% and from

TABLE 3

Comparison of two methods for determining total nucleic-acid-phosphorus of the terramycin-sensitive and -resistant *Streptococcus lactis* 10A

Culture	Incubation time	Total[a] nucleic acids	Nucleic Acid[b] calculated	Nucleic Acid[c] found
	(hr.)	*(milligrams per gram dry cells)*		
Sensitive	6	50.0	4.85	1.56
Resistant	6	35.4	3.43	1.25
Sensitive	12	33.5	3.25	1.28
Resistant	12	28.3	2.76	0.95
Sensitive	24	35.6	3.44	1.47
Resistant	24	33.7	3.26	1.19

[a] From Table 2. Determined on the basis of their ribose and deoxyribose content.
[b] Theoretically, ribonucleic acid of yeast contains 9.5 to 9.64% phosphorus, and deoxyribonucleic acid of thymus contains 9.89% phosphorus (12, 13).
[c] From Table 1.

2.8 to 3.5%, respectively. This difference between the two studies may be due to physiological differences between *S. lactis* and *S. pyogenes* or to the different methods used in obtaining the nucleic acid values. Nevertheless, there was agreement between the ratios of deoxyribonucleic acid to total nucleic acid found in *S. lactis* and in *S. pyogenes*. In the present work, deoxyribonucleic acid constituted 17 to 28% of the total nucleic acid content of *S. lactis*, compared to 10 to 30% in *S. pyogenes* (13). Also, Cohen (5) has reported that normal *E. coli* cells synthesize three times as much ribonucleic as deoxyribonucleic acid.

Previously, it was observed that the resistant strain utilized P^{32} at a slower rate than the normal strain (15). Also, Miura *et al.* (8) have reported that the inhibitory effect of aureomycin and terramycin on staphylococci is related to their inhibition of the transfer of high-energy phosphate bonds. On the basis of the present results, it would appear that during the development of antibiotic-resistance, the phosphorylation reactions of the parent *S. lactis* organisms were affected, and that the emerging mutant strain was physiologically incapable of utilizing as much phosphorus for nucleic acid synthesis as its parent strain.

Boivin (3) has reported that nucleic acids, combined with proteins, constitute an essential part of all animal and plant cell-nuclei and play an important part in the physiological functioning of the cells. The differences in the nucleic acids metabolism of the sensitive and resistant strains as found in this study might be associated with the physiological differences observed earlier (1, 14, 15) between the two strains.

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USE OF THE SELECTION INDEX METHOD TO ACCOUNT FOR THE NUMBER OF DAUGHTERS AND RECORDS IN PROGENY-TESTING A DAIRY BULL

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SUMMARY

Factors have been developed for taking account of the number of daughters and records in the progeny-test of a dairy bull. They are obtained by evaluating the regression of half the sire's additive genetic value on a function which compares the bull's daughter level to an expected daughter level. An extract from the table of the factors is given.

The selection index method of ranking animals according to predicted values of their genetic merit has been proposed on many occasions [Hazel (2) and Lush (4)]. Although it is a method which is theoretically simple, its application sometimes involves cumbersome algebraic expressions. In such cases, practical usage demands simplification, usually through extensive approximations. This paper outlines the process of such a series of approximations used in New Zealand when ranking dairy bulls, and shows how an unwieldy formula was reduced to a table of working values.

The progeny test of dairy bulls in New Zealand since 1950 has been based on comparing a bull's daughter average with the daughter average that would be expected in a herd of the production level in which the bull has been used. Most of the 3,000 proofs published annually in the Sire Survey (7), and earlier, are one-herd natural service proofs and, as such, are based on widely differing numbers of daughters and lactations. This paper shows how the proof has been amended to take account of these numbers, and discusses similar amendments for proofs of bulls used in artificial breeding. A table is given showing the factors used for making these amendments.

Difference from expectancy. Prior to the changes which this paper reports, bulls have been ranked by an expression known as the difference from expectancy. In computing this, the butterfat average for a bull's daughters is calculated each year, after age-correcting the records of the 2- and 3-yr.-olds. The average of the mature cows (ages 4–9 yr.) in the same herd, excluding any of the bull's daughters, is also calculated. From the latter, an expected daughter average is obtained from a regression line which has been estimated on many occasions for all proven bulls.

$$E\,(\text{daughter average}) = 76 + .75\,(\text{mature cow average}),$$

E being expectation over sires. The difference from expectancy for a bull is

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then the difference between his actual daughter average and this expected daughter average, namely

$$D.F.E. = \text{daughter average} - E\ (\text{daughter average}).$$

When a bull has daughters milked in more than 1 yr., the yearly values of this expression are averaged, weighted by the number of daughters milked each year, so that

$$\begin{aligned} D.F.E. &= \bar{d} - 76 - .75\bar{m} \\ &= \sum_i (d_i - .75 n_i \bar{m}_i)/L - 76 \end{aligned} \tag{1}$$

where L is the total number of daughter lactations in the proof, $\sum d_i$ is the sum over years of year totals for daughters, n_i is the number of daughters in the proof in year i, and $\bar{m}_i$ is the mature cow average in year i.

In publishing the proofs, the numbers of daughters and records in each proof were and are still shown; and the proofs used to be broadly classified as Preliminary, Intermediate, or Final, according to these numbers. This enabled the reader to assess the reliability of the proof for any bull, according to his own judgment of the relative importance of the number of daughters and records. This subjective method of weighing the results has now been replaced by factors which convert the difference from expectancy into an index now published as a rating.

Regression coefficients as factors. The object of sire selection indices is to rank bulls according to their breeding values. The breeding value of a bull includes both additive and nonadditive genetic parts, and by using a suitable mathematical model a prediction of the additive genetic part can be obtained by finding the regression of the bull's additive genetic value on a function of his daughters' records. Suppose this regression is

$$E(S) = \alpha + \beta\ (D.F.E.) \tag{2}$$

where S is the additive genetic value of a bull, $D.F.E.$ is the difference from expectancy, and E is expectation over the population of bulls. For ranking purposes, the α can be omitted, and since the additive genetic contribution of a bull to his daughters is $S/2$, the estimate of this which is suitable for ranking purposes is $\beta(D.F.E.)/2$. This is the estimated additive genetic superiority of the bull's daughters over those of an average bull, out of average dams in both cases.

Thus, $\beta/2$ is the factor used to correct the difference from expectancy into an index, now published as a rating. β would be the same for all bulls if they all had the same pattern of daughter and record numbers, and ranking of $D.F.E.$ alone would then suffice. This, however, is not the case. Most bulls in New Zealand are one-herd sires initially with few daughters and records in their proofs, the numbers increasing after the bull has been used for a year or two. These numbers affect the regression coefficients, and the problem was to develop an expression for β, then simplify it and reduce it to a workable form, in terms of the number of daughters and records.

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Mathematical model. The mathematical model used for the record of the kth daughter by the jth sire made in the ith year of the sire's proof was

$$d_{ijk} = \mu + \frac{1}{2}S_j + \frac{1}{2}D_{jk} + G'_{jk} + P_{jk} + Y_{ij} + e_{ijk} \qquad (3)$$

where μ is the mean yield of all daughters;

S_j is the additive genetic value of the jth sire;

D_{jk} is the additive genetic value of the dam of the kth daughter of the jth sire;

G'_{jk} is the additive genetic value of the daughter over and above $\frac{1}{2}(S_j + D_{jk})$, due to Mendelian sampling;

P_{jk} represents nonadditive genetic effects and permanent environmental effects peculiar to the daughter;

Y_{ij} is the effect of the ith year within the herd in which the jth bull was used; and e_{ijk} is the residual error peculiar to this record.

All terms in the model, except μ, were assumed to be random variables with zero means; the variances of S_j and D_{jk} were assumed equal, to σ_g^2, and that of G'_{jk}, was taken as $\sigma_g^2/2$ and the variances of P_{jk}, Y_{ij}, and e_{ijk} were taken as σ_p^2, σ_Y^2, and σ_e^2, respectively. Each set of terms (the S's D's ...) was considered to be a set of uncorrelated variables, each set being independent of the other.

These assumptions mean that the breeding value of a bull is uncorrelated with the breeding value of the cows to which he is mated; they also imply that the genetic variance among the bulls a farmer uses in his herd is the same as that among his cows, and that this is the same for all farmers. With one-herd bulls these assumptions will be untrue to the extent of the consistency with which a farmer can pick bulls as good as or better than the cows in his herd. The assumptions also imply that the effects on production due to years are uncorrelated, which is untrue when there are any carry-over effects from one year to the next; furthermore, it would probably be more correct to treat year effects as fixed rather than random. Despite these and no doubt other weaknesses in the model, it is expedient from a mathematical viewpoint to introduce no further terms.

When a sire's proof extends over more than 1 yr. (as is frequently the case), the required regression term involves correlations that utilize the following parameters:

$$r = \text{repeatability} = (\sigma_g^2 + \sigma_p^2)/\sigma^2$$
$$h = \text{within-herd heritability} = \sigma_g^2/\sigma^2$$
$$t = \text{"seasonability"} = \sigma_Y^2/\sigma^2$$

σ_g^2 is the additive genetic variance within a herd, so that h as here defined is heritability in the "narrow sense"; σ^2 is the variance of a record d_{ijk}. The following table shows the appearance of these parameters in correlations between various pairs of records that can form part of the difference from expectancy expression.

Interpretation of this table is exemplified by considering the terms in the first

TABLE 1
Correlations between all possible pairs of records that can exist in a proof

Year in which record is made	Animals making records: Daughters j	Daughters j'	Mature cows k	Mature cows k'	Daughter Dam (jk)	Daughter Dam (jk)	Daughter	Mature cow, not the daughter's dam
i	$\leftarrow h/4+t \rightarrow$		$\leftarrow t \rightarrow$		$\leftarrow h/2+t \rightarrow$		$\leftarrow t \rightarrow$	
	$\uparrow r$	$h/4 \nearrow \swarrow$	$\uparrow r$		$\uparrow r$	$h/2 \nearrow \swarrow$		
i'	$\downarrow$		$\downarrow$		$\downarrow$			

cell; the correlation between the record of daughter j in year i and its record in year i' is r; the correlation between its record in year i and that of its half-sib, j', in the same year is $(h/4 + t)$; and the correlation between half-sib records made in different years i and i', is $h/4$.

Assumptions. The first assumptions applied to the model to obtain the regression were

(i) that one-third of the daughters milked in any year have their dams milked in the same year,

(ii) that no daughters milked in any year of the proof have their dams milked in some other year of the proof, and

(iii) that when a daughter goes out of production it does not return to the herd again.

With these assumptions and parameters, it can be shown from (1), (2), and (3) that β can be obtained from the equation

$$\frac{hL^2}{2\beta} = (1\text{-}r\text{-}t)L + 0.5h(L^2 - \sum_i n_i^2/m_i) + (r\text{-}h/4)\sum_j l_j j^2 + \sum_i n_i^2\,[t + 9(1\text{-}t)/m_i]/16 + \frac{9r}{16m_i}\sum_{i \neq i'} n_i n_{i'} \frac{p_{ii'}}{m_i m_{i'}}, \quad (4)$$

where L is the total number of lactations in the proof, l_j daughters have had exactly j lactations, n_i daughters are milked in vear i, m_i mature cows (not being daughters) are milked in year i, and $p_{ii'}$ mature cows are milked in both years i and i'.

Further assumptions were needed in order to reduce (4) to a simplified expression for β. These included using estimates of some of the parameters, thus leading to an estimate of β. The added assumptions were

(iv) that the number of mature cows (not daughters) in the herd is the same every year, and that this number is 40, and

(v) that estimates of the parameters are $r = .45$, $h = .25$, and $t = .20$.

The expression for β then reduced from (4) to

$$1/\hat{\beta} = 2.8/L + 1/2 + \frac{3.1}{L^2}\sum_j l_j j^2 + \frac{.1775}{L^2}\sum_i n_i^2 + \frac{.0506}{L^2}\,\Sigma_{i \neq i} n_i n_{i'}\, p_{ii'}\,/40 \quad (5)$$

The assumed value of n_i is 40; thus, $p_{ii'}/40$ when $(i-i') = 1$, is the fraction of the mature cows in a herd that are milked in two consecutive years. From the New Zealand Dairy Board (5), an estimate of this fraction was obtained as 70%, giving values for the fraction of .50 and .34 when the difference between i and i' is 2 and 3, respectively. With these values, expression (5) reduced to a function dependent on the number of daughters a sire has, and the pattern of lactations which they have made over the years of the proof. Such an expression could be computed for each individual sire but, from a practical viewpoint, this is quite unfeasible, so further simplification was sought.

Simplifications leading to working values. First, use was made of certain relationships existing among some of the terms already defined, namely

$$\sum_j d_j = N, \text{ and } \sum_j n_i = \sum_j l_j j = L.$$

Two new sets of parameters were defined, dealing with the pattern of culling in a herd: r_2', r_3', and r_4' . . . were defined as the fraction of 2-, 3-, and 4-yr.-olds, respectively, retained in a herd from one year to the next, so that $(1-r_2')$ for example, is the culling rate for two-yr.-olds; p_1, p_2, and p_3 . . . were taken as the proportions of the total number of daughters in the complete proof, which were in the proof at the end of the 1st, 2nd, 3rd . . . yr. of the proof, respectively. These r''s and p's completely specify the culling that has taken place in a herd, and they were substituted into expression (5) in place of the l_j's. Although it is not true that the same culling rates on an age basis do apply in all years of a proof and for all herds, this is an expedient and practical form of simplification.

At this stage, the expressions for $\hat{\beta}$ were considered separately for 1-, 2-, 3-, and 4-yr. proofs. For a 1-yr. proof, the expression is

$$1/\hat{\beta} = \frac{5.9}{L} + .6775, \tag{6}$$

and this can be computed directly for any value of L.

When there are just two years' daughters in a proof

$$1/\hat{\beta} = \frac{12.1}{L} - \frac{6.2}{pL} + .6775 - \frac{.2841 p_1(1-p_1)}{p^2}, \tag{7}$$

where p is the average number of lactations per daughter,

$$\text{i.e., } p = L/N, \tag{8}$$

$$\text{and } p = 1 + r_2' p_1, \tag{9}$$

by the definition of r_2' and p_1. For seven different values of p_1 (fraction of the total number of daughters in the proof that were in it the 1st yr.) ranging from 1.0 to 0.2, and for seven values of r' (fraction of 2-yr.-olds retained in the herd each year) ranging from 0.0 to 0.5, values of p were calculated from (9). For each of these, values of p, $\hat{\beta}$ and N were expressed in terms of L from (7) and (8) and these were then calculated for all values of L from 6 to 45. For any given

values of L and N it was found that the values of $\hat{\beta}$ for different values of p differed only at the third decimal place. The values of $\hat{\beta}$ were, therefore, summarized in a two-way table according to N and L.

This same procedure was followed for calculating $\hat{\beta}$ for proofs spreading over 3 and 4 yr., extending the values of L in these cases to 90 and 150, respectively. The "smoothing" of the tables became progressively a little more difficult, due to the gradations in p (the average number of lactations per daughter) not being so fine as in the 2-yr case. The sets of values for the p's and r's that were used were by no means exclusive, but they covered a wide range, from no culling to total culling, and from the case of assuming all daughters in a proof being in it the 1st yr. to where only a few of them are. No highly irregular values of $\hat{\beta}$ appeared.

There were now four tables of $\hat{\beta}$ values, according to values of N and L, dependent on whether the proof was based on 1, 2, 3, or 4 years of lactations. On comparing the tables, it was found that for any specified values of N and L, the maximum differences among the $\hat{\beta}$'s of the four tables was 0.02, so that amalgamating the tables entailed only negligible errors. For the same reason, it seemed unnecesary to carry out the calculations for proofs spreading over more than 4 yr.

Values of $\hat{\beta}$ had been computed in each of the four tables for values of L taken at intervals of one from 6 to 20, intervals of two from 20 to 40, and thereafter at intervals of five. They changed with increasing slowness for large values of L, so the intervals were widened in the amalgamated table, taking two groups as 31-33 and 34-37, and subsequently grouping round L-values of 40, 45 . . . etc. Beyond 82, the L's were grouped by 15's, centering on 90, 105, ... A similar type of grouping was used for the daughter numbers, starting at 25; by one's up to 25, then 26-28, 29-31, and thereafter by 5's, centering on 35, 40, ... These groupings seemed favourable; beyond 30, lactation numbers assume much importance only in multiples of five, and beyond 25 the importance of daughter numbers can not be assessed by individual ones. The result was a comparatively smooth table without any undue repetition of terms.

Consideration of assumptions. It is interesting to consider the assumptions used in arriving at these results. The values of 1/3 in assumption (i) is taken from the New Zealand Dairy Board's Annual Report (5); although assumption (ii) is in direct negation of (i), the fraction would, at most, be small and contribute little to the final result. Assumptions (iii) and (iv) are not true in practice, but practical necessity demands some simplifications, and these are not unreasonable. The average herd size in New Zealand is 60 cows, and although 40 might, therefore, be a little high for the average number of mature cows, a smaller value has little effect on the factors; using 25, decreases them by about 3%. The values used for r and h are in line with those in general use; subsequent work of Castle and Searle (1) has shown that more appropriate values might be $r = .60$, and $t = .10$, but the effect of these changes would be small. Using a constant value for p_{ir}/m_i for all herds has the same weaknesses as assumptions (i) and (iv), but

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TABLE 2

Extract from table of factors used for converting difference from expectancy to an index

Number of daughters in progeny-test *(N)*	Number of records in progeny-test *(L)*																			
	6	7	8	9	10	12	14	16	18	20	25	30	35	40	45	50	60	70	80	90	100
6	.30	.31	.32	.33	.34	.36	.37	.38	.39	.40	.41										
7		.33	.34	.35	.36	.37	.38	.39	.40	.41	.43										
8			.35	.36	.37	.39	.40	.40	.42	.43	.45	.47									
9				.37	.38	.40	.41	.42	.43	.44	.46	.48	.50								
10					.39	.41	.43	.44	.45	.46	.48	.50	.51	.52							
12						.43	.45	.46	.47	.48	.50	.51	.53	.54	.55	.56					
14							.46	.47	.49	.50	.52	.53	.55	.56	.57	.58					
16								.48	.50	.51	.53	.55	.56	.57	.58	.59	.61				
18									.50	.52	.54	.56	.57	.58	.59	.60	.62	.63			
20										.51	.55	.57	.59	.60	.61	.62	.63	.65	.66		
25											.55	.58	.61	.61	.62	.63	.65	.66	.67	.68	
30												.57	.62	.63	.64	.65	.67	.68	.69	.70	.71
35													.59	.63	.65	.66	.67	.69	.70	.71	.72
40														.60	.63	.66	.69	.70	.71	.71	.73
45															.61	.64	.69	.71	.71	.72	.72
50																.62	.68	.72	.73	.73	.74
Artificial breeding $L/(L+15)$	.29	.32	.35	.38	.40	.44	.48	.52	.55	.57	.63	.67	.70	.73	.75	.77	.80	.82	.84	.86	.87

the only feasible procedure for terms of this nature which vary so much from herd to herd is to use average values, and apply them universally to all herds.

Factors for A.B. bulls. The development of the regressions as given above is for bulls used in natural service in just one herd. Those used in artificial breeding have daughters in many herds and this fact had to be considered in developing the regression factors for such bulls. It was assumed that every daughter is in a different herd, and that the proof is based on just 1 yr.'s records. In many cases this is not true, but it is (or very nearly so) at the time of a bull's first proof, when his first crop of daughters have made their first records. This is probably the most important proof of all for a bull being used in artificial breeding, because his fate will be decided upon it—continued use in the stud or retirement to the meat works.

The mathematical model used was very similar to that used in the case of natural-service bulls, namely,

$$d_{jk} = \mu + \frac{1}{2} S_j + \frac{1}{2} D_{jk} + G'_{jk} + P_{jk} + H_k + e_{jk}$$

where d_{jk} is the record of the jth bull in herd k. The terms in this model are the same as previously except that Y_{ij}, the effect due to year i in the herd in which the jth bull was used, is now replaced by H_k, the effect due to herd k. The effect due to the ith year is not needed here because of assuming the proof is based on just 1 yr.'s records. Since a bull used in A.B. has daughters in many herds, a herd effect term, H_k, is needed; it was not included specifically in the model (3), for one-herd sires having natural proofs, because such proofs are based on daughters all in the same herd, and in this situation the herd-effect is essentially included in the terms $\mu + y_{ij}$. With sires used in artificial breeding, H_k is assumed a random variable with zero mean and variance σ_H^2. Taking $\sigma_S^2 = \sigma_D^2 = \sigma_g^2$, as has been done, assumes that genetic variance among sires is the same as that among cows within a herd, an assumption that may or may not be true. The same parameter values were used as in the natural service case, with the additional one that σ_H^2 is 30% of the total variance, as in Castle and Searle (1).

The difference from expectancy in this situation can be expressed as

$$D_j = \sum_k (d_{jk} - .75\,\bar{m}_{jk})/L - 76$$

where $\bar{m}_{jk}$ is the mature cow average in Herd k. With the assumptions that have been made, the regression of $\frac{1}{2} S$ on D reduces to

$$\frac{L}{L + 15.456}$$

which, for practical usage, can be expressed as

$$\frac{N}{N + 15}$$

where N is the number of daughters (assumed equal to the number of lactations). It is interesting to note that by evaluating the intra-sire regression (for A.B. sires) of daughter average on contemporary average, Henderson in the United States (3) and Robertson in England (6) obtained values $N/(N+12)$ and $N/(N+18)$, respectively.

Extract of table of factors. The accompanying table is an extract of the complete table of the $\hat{\beta}$ values, the regression of the "difference from expectancy" on one-half the sire's additive genetic value. The difference from expectancy is converted to an index by multiplying it by the appropriate value in this table, dependent upon the number of daughters and lactations in the proof. The bottom line of the table shows the factors for A.B. bulls, where it is assumed that the number of daughters equals the number of lactations. As is to be expected, the factors increase at an ever-decreasing rate as the number of daughters, or the number of lactations, or both, increases. At the lower daughter numbers, increasing the number of daughters increases the factor more than does increasing the number of lactations from the same daughters. This effect acts in reverse, but not so strongly, when there are more than about 25 daughters. The added lactations then imply more information about an already-substantial number of daughters and, if this implies an added number of years in the proof, it means there is then more information about the herd level. These trends show that the first importance is to sample the bull (i.e., get a reasonable number of daughters, about 20) and then sample the environment by getting more lactations from them.

These factors are now being used in the preparation of progeny-test proofs in New Zealand, the index $\hat{\beta}$ (*D.F.E.*) being termed a rating.

ACKNOWLEDGMENT

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A COMPARISON OF ORCHARDGRASS-LADINO CLOVER AND ORCHARDGRASS AS PASTURE FOR MILKING DAIRY COWS[1]

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SUMMARY

This study indicated higher average daily production of 4% FCM and greater returns over concentrate and fertilizer costs from a mixed pasture of orchardgrass and Ladino clover than from orchardgrass heavily fertilized with nitrogen. Differences in production of FCM between the two pastures averaged 5.0, 3.1, and 2.8 lb/cow/day for years 1955, 1956, and 1957, respectively. Likewise, differences in average returns over concentrate and nitrogen costs were \$0.31, \$0.23, and \$0.18/cow/day for the same years. The progressive decline in differences in production and returns between pasture treatments by years appeared to be related to a similar decline in the percentage of clover in the mixed stand. Returns per acre were greater for the grass and clover mixture by \$47.33 in 1955 (a short pasture season) and by \$55.85 in 1956. In 1957, returns from orchardgrass pasture exceeded those from the mixed pasture by \$5.61 per acre. However, 41 more cow days were required per acre to produce this added return from orchardgrass.

Agronomic yield studies of grasses and grass-legume associations have been conducted under many conditions. In general, the grass-legume mixtures have yielded more dry matter per acre than pure grass stands. However, numerous workers (7, 9, 10) have shown greater dry matter production by pure grass stands heavily fertilized with nitrogen. Very little work has been published concerning the relative nutritive value of grass versus grass-legume pastures for ruminants.

Heinemann and Van Keuren (6) and Van Keuren and Heinemann (12, 13) have reported higher average daily gains by beef and more pounds of beef per acre from grass-legume mixtures than from pure grass pastures. In 1954, the pounds of beef produced per acre from Ladino-orchardgrass pasture exceeded beef production from tall fescue by 26% and from orchardgrass by 76%. The average daily gains from Ladino-orchardgrass were 25% higher than from tall fescue and 39% higher than from orchardgrass in the same comparisons. In 1955, the mixed grass and legume stands again produced more beef per acre and higher average daily gains than did the grass pastures. However, the differences were not so great during the 2nd yr. This was attributed to a very marked reduction in the percentage of clover in the mixed stand. The grass plots were fertilized each year with 200 lb. of nitrogen per acre. The grass and legume

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plots were fertilized with 125 lb. of nitrogen per acre in 1954 and 100 lb. per acre in 1955.

The Oregon Agricultural Experiment Station (1) has similarly reported more beef production per acre from grass and Ladino clover than from all-grass pastures heavily fertilized with nitrogen. The monetary returns per acre were also greater for the grass-legume pasture.

McVey and Mott (8) obtained more beef production per acre from a mixture of bluegrass and birdsfoot trefoil than from bluegrass fertilized with 120 lb. of nitrogen per acre.

Blaser *et al.* (4) found lower daily weight gains of beef grazing orchardgrass or tall fescue fertilized with nitrogen than of animals grazing the same grasses with Ladino clover and not fertilized with nitrogen. The weight gains per acre were slightly higher for the animals grazing the pure grass pastures, but the differences were not great enough to pay for the nitrogen fertilizer used. The yearly rate of fertilization was 216 lb. of nitrogen per acre.

In a three-year study, Seath *et al.* (11) found that orchardgrass-Ladino pasture ranked first in monthly persistency of milk production (98.3%), followed in order by Kentucky 31 fescue-Ladino (89.2%), Kentucky bluegrass–Ladino (87.2%), and a straight stand of Kentucky 31 fescue (81.4%). The straight fescue was fertilized with 200 lb. of ammonium nitrate yearly. Under these conditions, the fescue alone produced the highest yearly yield of TDN per acre (2,327 lb.); whereas, the three other pastures yielded about the same, varying from 1,726 to 1,789 lb. per acre.

Wagner (14) has shown that Ladino clover fixed on the average more than 150 lb. per acre annually of utilizable nitrogen when grown with orchardgrass or tall fescue.

EXPERIMENTAL PROCEDURE

The primary objective of this experiment was to compare milk production of cows from orchardgrass–Ladino clover pasture with that from an all-orchardgrass pasture liberally fertilized with nitrogen.

Pasture establishment and fertilization. In the fall of 1954, three replicated plots each of orchardgrass–Ladino clover and orchardgrass were established for this experiment. Each plot was 0.94 acre. Akaroa orchardgrass was sown at the rate of 12 lb. per acre in the grass plots and 10 lb. per acre in the grass-legume association. Certified Ladino clover was sown at the rate of 2 lb. per acre in the latter. The land on which these seedings were established was Puyallup fine sandy loam. It had been clean-cultivated for 2 yr. to eradicate previous plant species.

Mixed fertilizer (10-10-5) was applied uniformly at the rate of 350 lb. per acre at seeding time. The subsequent fertilizer treatments by years are shown in Table 1. In 1955 and 1956, the P_2O_5 and K_2O were split equally between a spring and fall application. In 1957, only a spring application was made because the project was concluded at the end of the grazing season.

Nitrogen was applied to the orchardgrass plots at the rate of 35 lb. N per application. The first application was in early spring and subsequent appli-

TABLE 1

Pounds of N-P_2O_5-K_2O applied per acre by years

Treatment	1955	1956	1957
Orchardgrass	120–130–65	210–125–62	210–64–32
Orchardgrass–Ladino clover	0–130–65	35–125–62	35–64–32

cations were made after each grazing. No nitrogen was applied to the grass-legume plots in 1955. However, in 1956 and 1957, 35 lb. of N were applied per acre to the grass-legume plots in early spring. The nitrogen was applied as ammonium nitrate by means of a mechanical spreader.

All plots were irrigated by sprinkling as needed to supply adequate moisture.

Animal and pasture management. The first cutting of all plots was harvested for silage in the spring of 1955 because of the preponderance of annual weeds. Therefore, the pasture phase of the experiment was not started until July of that year. All plots were strip-grazed throughout the remainder of that season and, also, during 1956 and 1957.

Adjacent plots of each pasture treatment were grazed simultaneously by two groups of lactating purebred Holstein cows during the 3 yr. However, the grazing management was varied slightly between years.

During 1955, two groups consisting of six cows each were paired as nearly as possible according to age, stage of lactation, and milk and fat production. The two groups were assigned at random to the two pasture treatments. Strips of fresh pasture (subplots of approximately 0.08 acre in size) were made available for grazing each day by means of a movable electric fence. Thus, 12 days were required to graze each pair of plots and 36 days to complete a grazing cycle. The cows were not excluded from the previously grazed subplots and were allowed to graze them until moved to the next plot.

At the end of each grazing cycle the animal groups were reversed in regard to the pasture treatments. Two and two-thirds grazing cycles were completed during 1955.

The 36-day grazing cycle used during 1955 was too long and the forage became too mature between grazing periods. Therefore, the size of the daily subplots was increased in 1956, so that only nine days were required to graze each plot and 27 days to complete a cycle. The length of the first grazing cycle was shortened to 16 days in order that all plots would be grazed at a more desirable stage of maturity. In addition, the electric fence was moved to provide fresh pasture twice daily instead of once daily, except for the first day on each plot. A full day's grazing was allowed on the first setting to avoid severe tramping of a restricted area.

Two groups of paired milking cows were used during 1956 in the same type of change-over design used in 1955. The number of cows in each group was changed at times during 1956, to adjust the grazing load to available forage. However, equal numbers were pastured on each treatment and the balance was maintained between groups as nearly as possible. Five and two-thirds grazing cycles were completed during 1956.

Under the fertilization program previously indicated, the total forage dry matter production of the orchardgrass plots during 1956 was significantly greater than that of the grass and clover plots (Table 2). Likewise, the amount of uneaten forage was considerably greater on the grass plots. Therefore, the experimental design was changed during 1957 to measure differences in total milk production per acre when both types of pasture were utilized to approximately the same degree.

Lactating cows were paired as in previous years, but only to the extent of the number grazing the grass-clover plots. Additional cows were added and removed as needed to approximate optimum pasture utilization. Also, animal groups were not reversed in regard to pasture treatments in 1957, but each group remained on the assigned treatment throughout the grazing season.

During all three pasture seasons, the cows were confined to the pastures night and day, except for approximately 2 hr. night and morning while they were at the barn for milking.

Changes in animal weights were ascertained in each of the 3 yr. During 1955 and 1956, the averages of two consecutive daily weights were determined at the beginning and end of each grazing cycle. The net weight gain or loss was then derived for both treatments from these average weights. In 1957, initial and final weights were determined by averaging three consecutive daily weights as animals were started and removed from the experiment.

Concentrate feeding. A simple concentrate mixture containing 15% crude protein was fed at the rate of 1 lb. per 7 lb. of 4% FCM. Adjustments in rate of concentrate feeding were made every six days during the 1955 season and at nine-day intervals during 1956 and 1957.

Observations and analyses. Milk weights for each milking were recorded to the nearest 0.1 lb. Composite milk samples were tested for butterfat at six-day intervals during 1955 and at nine-day intervals during 1956 and 1957. Weights of concentrates fed and refused were recorded to the nearest 0.1 lb. The amounts of fertilizer applied in pounds and dates of application also were recorded. Representative samples of concentrates fed were analyzed for proximate composition by standard A.O.A.C. procedures (2).

Dry matter yields of the pastures were computed from mowed samples taken from successive strips before the cows were allowed to pasture. After the cows were moved to the next replicate, comparable strips were mowed again to determine the amount of dry matter apparently consumed. The Gravely mower used to make these clippings left very little trampled forage after grazing.

The percentages of grass and clover in both pasture treatments were determined on a dry weight basis from hand separations of samples of clipped forage taken for dry matter yields.

Significances of differences in production of 4% FCM between pasture treatments were determined on the data from four grazing cycles during 1956, by an analysis of variance according to the methods of Federer (5). Similar statistical analyses could not be applied to other cycles in the 1955 and 1956 seasons because of experimental design.

RESULTS AND DISCUSSION

The dry matter yields of the pastures and the dry matter consumption by the grazing animals are summarized in Table 2. Correlations of dry matter yields, dry matter consumption, and milk production are being reported in another publication (3).

Variations in the percentage of clover in the stands and the weighted averages for the 3 yr. are shown in Table 3. A yearly decline in the percentage of clover in the mixed stand is very evident. Differences in dry matter production between the grass and the grass-clover appear to be related to the yearly decline in the clover stand and a concomitant decline in nitrogen fertility of the mixed stand.

A nitrogen deficiency became apparent in the orchardgrass-Ladino plots after the first grazing cycle in 1957. This was evidenced by the yellowish color of the grass and reduced rate of growth as compared to grass in the nitrogen fertilized plots. Data in Table 2 indicate higher dry matter yields of both treatments in 1957 than in 1956, in spite of the nitrogen deficiency apparent in the grass-clover plots. These yearly differences were the result of differences in spring growing conditions. Spring forage production was low in 1956, following a long, severe winter, but very abundant in 1957 when growing conditions were very favorable.

The consumption of dry matter in 1955 was significantly higher on grass and clover than on grass alone, even though there was no significant difference in the total dry matter production between treatments. Dry matter production in 1956 was significantly higher for the pure grass plots, but there was no apparent difference in consumption between the two treatments. In 1957, both the dry matter production and the dry matter consumption were significantly higher for the pure grass treatment. In all 3 yr., the percentages of available dry matter consumed by the grazing animals were higher for the orchardgrass-Ladino treatment than for the pure grass treatment. These differences indicate that the grass and clover combination was more palatable.

As has been previously indicated, the rate of stocking was varied between treatments during 1957 so that the available forage from both treatments would be used to the same extent. That this was nearly accomplished is indicated by 67 and 70% consumption of available dry matter from the orchardgrass and orchardgrass-Ladino pastures, respectively.

Variations in the average production of FCM by two groups of cows grazed alternately on orchardgrass-Ladino clover and orchardgrass pastures during 1955 and 1956 are shown graphically in Figures 1 and 2. An increase in milk production is evident when either group was changed from grass pasture to the mixed grass and clover. Each bar in the graph represents the average production by the group while grazing one plot. Three bars represent a complete grazing cycle.

Inasmuch as the experimental design was changed during 1957 so that unequal numbers of animals were pastured on the two treatments, it was not possible to present the production data graphically for that year in the same manner as indicated above.

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TABLE 2

Dry matter yields and apparent dry matter consumption of grazing animals by years

	1955			1956			1957		
	Orchard-grass	Orchard-grass–Ladino clover	Difference	Orchard-grass	Orchard-grass–Ladino clover	Difference	Orchard-grass	Orchard-grass–Ladino clover	Difference
D.M. production/acre *(lb.)*	4,090	4,420	330	8,430	7,160	1,270**	9,860	8,050	1,810**
D.M. consumed *(lb.)*	3,200	3,770	480**	5,430	5,280	150	6,590	5,690	900**
D.M. consumed *(%)*	78	85		65	74		67	70	

** Statistically significant at the 1% level.

TABLE 3

Percentage of clover in stands of orchardgrass and orchardgrass–Ladino clover pasture by years (percentages on oven-dry basis)

	1955		1956		1957	
	Orchardgrass	Orchardgrass–Ladino clover	Orchardgrass	Orchardgrass–Ladino clover	Orchardgrass	Orchardgrass–Ladino clover
Range *(%)*	0–4	43–53	0–3	22–39	0–5	3–26
Weighted av. *(%)*	2	47	0	30	3	11

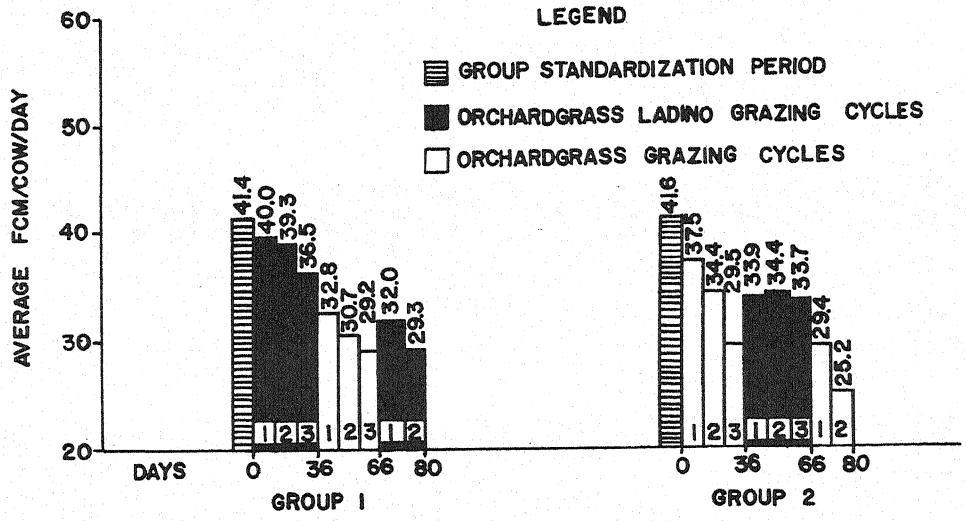


FIG. 1. Variations in average production of FCM by cows on orchardgrass–Ladino clover and orchardgrass pastures during the 1955 season. (Numbers in bars denote successive plots grazed within each pasture cycle.)

An analysis of variance was made on a portion of the milk production data during 1956, to test the significance of differences in milk production between cows while grazing orchardgrass-Ladino clover and orchardgrass alone. Data from the first and last grazing cycles could not be used in this analysis, because the number of days and the number of animals used were not the same as in the intervening cycles. Also, the data from the first plots grazed after each reversal were not used, since these were considered as change-over periods. The period covered by this analysis was from May 30 through September 15 and involves the data from four complete grazing cycles and three group reversals under each treatment. There were five milking cows per group during this period.

The results of this analysis are in Table 4. The greater production of 4% FCM by the cows while grazing orchardgrass–Ladino clover was very highly significant ($P = < 0.0005$).

TABLE 4

Analysis of variance to test the significance of differences in production of 4% FCM by cows grazing alternately orchardgrass–Ladino clover and orchardgrass alone, from May 30 through September 15, 1956

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	39	782,237.46	
Periods	1	169,572.49	
Cows within periods	18	517,343.38	
Subperiods	2	46,765.75	
Treatments within periods	2	36,786.03	
Treatments	1	33,744.49	33,744.49**
Treatments × periods	1	3,041.54	
Error	16	11,769.81	735.61

** $P = < .0005$.

In comparing the milk production and forage production and consumption data by years, it is interesting to note that during 1956 cows produced more milk while grazing the grass-clover mixture, in spite of the fact that more total forage dry matter was consumed from the grass plots. However, differences in dry matter consumption under the two treatments were not significant during that year.

Summaries of the production of 4% FCM and of the economic returns from orchardgrass–Ladino clover and orchardgrass pastures for the 3 yr. are in Table 5. The summaries for 1955 and 1956 do not include that portion of the data applicable to the change-over periods as previously described. Inasmuch as each animal group was confined to one pasture treatment throughout 1957, the data from all plots were included in the summary for that year.

Average daily production of FCM per cow while grazing orchardgrass–Ladino clover exceeded that from cows while grazing orchardgrass during all 3 yr. The differences in FCM production between the two pasture treatments were 5.0, 3.1, and 2.8 lb. per cow per day for the years 1955, 1956, and 1957, respectively. Similarly, the average returns from grass and clover pasture exceeded those from grass alone by \$0.31, \$0.23, and \$0.18 per cow per day for the 3 yr.

The average returns per acre from the orchardgrass-Ladino plots exceeded returns from orchardgrass during 1955 and 1956, but fell slightly below the grass

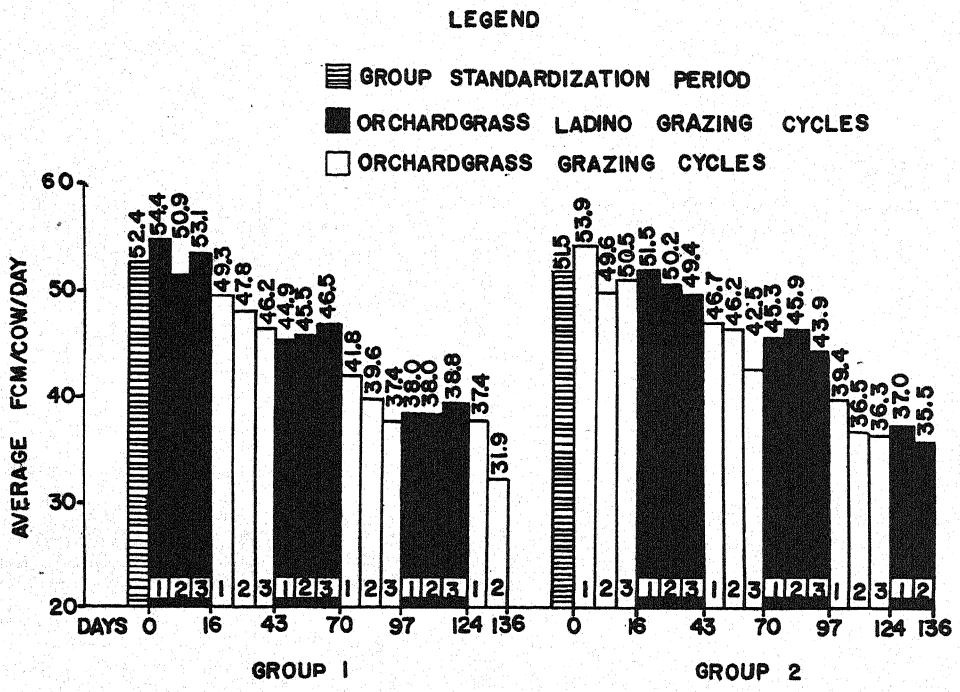


FIG. 2. Variations in average production of FCM by cows on orchardgrass–Ladino clover and orchardgrass pastures during the 1956 season. (Numbers in bars denote successive plots grazed within each pasture cycle.)

TABLE 5

Summary of 4% FCM produced, concentrates fed, ammonium nitrate used, and economic returns per acre from orchardgrass and orchardgrass–Ladino pasture by years

Item	1955			1956			1957		
	Orchard-grass	Orchard-grass–Ladino	Differ-ence	Orchard-grass	Orchard-grass–Ladino	Differ-ence	Orchard-grass	Orchard-grass–Ladino	Differ-ence
Cow days	153	153	.....	248	248	.....	309	268	41
4% FCM *(cwt.)*	46.52	54.18	7.66	105.18	112.78	7.60	118.09	109.93	8.16
Av. FCM/cow/day *(lb.)*	30.4	35.4	5.0	42.4	45.5	3.1	38.2	41.0	2.8
Concentrate consumed *(lb.)*	719	802	83	1,569	1,603	34	1,727	1,618	109
Ammonium nitrate used *(lb.)*	372	.....	372	628	106	522	638	106	532
Milk value @ $4.25/cwt	$197.71	$230.27	$32.56	$447.02	$479.32	$32.30	$501.89	$467.20	$34.69
Concentrate costs @ $70/T	$ 25.17	$ 28.07	$ 2.90	$ 54.90	$ 56.11	$ 1.21	$ 60.44	$ 56.63	$ 3.81
Nitrogen costs @ $95/T	$ 17.67	$ 0.0	$17.67	$ 29.81	$ 5.05	$24.76	$ 30.32	$ 5.05	$25.27
Return over costs of concentrate and nitrogen	$154.87	$202.20	$47.33	$362.31	$418.16	$55.85	$411.13	$405.52	$ 5.61
Av. return/cow/day	$ 1.01	$ 1.32	$ 0.31	$ 1.46	$ 1.69	$ 0.23	$ 1.33	$ 1.51	$ 0.18

plots in 1957. As indicated in Table 2, considerably more dry matter remained on the orchardgrass plots after grazing than on the grass-clover plots. This was particularly true during 1956. Whether this ungrazed dry matter could have been utilized economically is problematical. In 1957, when more cows were allocated to the orchardgrass treatment to use the available forage of both treatments to the same extent, it was found that 41 more cow days per acre were required for the orchardgrass treatment. Additional labor and investment costs, not previously taken into account in calculating the returns shown in Table 5, probably would nullify these apparently greater returns. Thus, it appears that greater production of milk per acre was obtained at the expense of reduced production per cow.

Only the costs of concentrates fed and of the ammonium nitrate applied to the pastures were taken into account in computing the returns from pasture. It is assumed that other costs would be comparable for both treatments, except as noted above during 1957. A value of $4.25/cwt for 4% FCM was used, because that was the approximate blend price for milk produced in the Puget Sound area during the summer of 1958. The prices used for concentrates and ammonium nitrate are the approximate prices farmers would pay for these commodities in the area.

The average gains and losses in body weight per cow day are shown in Table 6. Inasmuch as weight changes were quite variable between years and between animals within treatments, their significance is questionable. However, during 1956 there was a definite tendency for cows to make greater weight gains while grazing pure orchardgrass than while grazing grass and clover.

TABLE 6

Average gains (+) and losses (−) in body weight per cow day by treatments and years (lb.)

Treatment	1955	1956	1957
Orchardgrass	−0.24	+1.26	+0.28
Orchardgrass–Ladino clover	+0.12	−0.29	+0.10

The formula for the concentrate mixture fed the cows was the same for all 3 yr. The average proximate analysis of the concentrate mixture was 9.6% moisture, 15.0% protein, 3.7% ether extract, 7.0% crude fiber, and 5.5% ash.

One cow bloated on two occasions in 1955 on the orchardgrass-Ladino pasture. The condition was relieved on both occasions by appropriate medication or by means of a stomach tube. These were the only instances of bloat in 1,547 cow days of grazing orchardgrass-Ladino pasture during the 3 yr. of the experiment. Both instances of bloat occurred during the first day's grazing of a new orchardgrass-Ladino plot when the cows had access to a full day's grazing at one setting. It seems likely that the system of strip grazing used (two settings per day) may have aided materially in the control of bloat.

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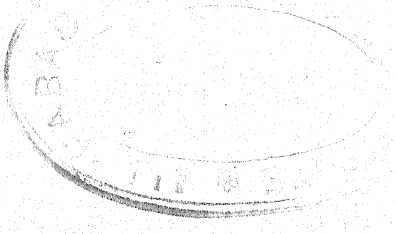
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A COMPARISON OF THE RELATIVE EFFICIENCY OF THREE PASTURE UTILIZATION SYSTEMS

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SUMMARY

Utilization of pasture forage by rotational grazing, strip grazing, and soiling, and the effect of these systems on milking dairy cows, was compared at Beltsville in 1954-56. Forage was produced on improved pasture typical of the area. Forage utilized per acre was measured as calculated TDN furnished to milking cows plus TDN removed as excess forage. Effect of the systems on the cows was measured by seasonal milk production and live weight changes.

Average amounts of forage utilized per acre were essentially the same for the two grazing systems. These values were significantly greater for both grazing systems than for the soiling system.

Neither milk production nor live weight changes per cow were significantly affected by the method of providing forage.

The legume content of forage from all areas was rapidly reduced during an extended dry period and remained low during the remainder of the experiment.

Conventional rotational grazing was distinctly the most desirable method of utilizing the forage produced under the conditions of this experiment, although it has been recognized that production of taller, more intensive crops and/or more wasteful management of rotational grazing, such as understocking or allowing forage to become more mature, will provide conditions offering a distinct advantage to strip grazing or soiling.

Three rather distinct pasture forage utilization systems have developed from pasture investigations, in an attempt to reduce the forage wastes that have typified set-stocking and continuous grazing of high-yielding pasture. Although the systems vary in detail and nomenclature between locations, they may be generally described as: 1. Rotational grazing (also known as paddock grazing), whereby the plots of a divided pasture area are grazed in succession for from five to ten days each. 2. Strip grazing (also known as break grazing, daily rationed grazing, and close folding), whereby a subplot sufficient for one day's grazing is assigned each day. 3. Soiling (also called zero grazing, mechanical grazing, and green chop feeding), whereby the forage is mechanically harvested and fed to cows in the barn or dry lot. The relative merits of these three utilization systems are, as yet, matters of no general agreement. Comparisons under a wide variety of situations will be required to assess their values with certainty.

Interest in strip grazing was initially stimulated by reports from Great Britain. Holmes *et al.* (9) in 1950 and Waite *et al.* (23) in 1952 reported marked increases in pasture yields in terms of cow-days, milk, and calculated starch equivalents from strip grazing as compared to rotational grazing. The greater efficiency resulted from the smaller acreage requirement of the strip grazing group. However, additional reports by the same authors, (10) and (24), respectively, showed the rotationally grazed group to have obtained an appreciably higher dry matter intake, indicating that the need for additional acres may have

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been less real than apparent. Similar increases in milk yield per acre through the reduced grazing wastes of strip grazing were reported by Procter *et al.* (17, 18).

Soiling has been practiced in this country and abroad for many years. However, soiling has been confined to specialized annual crops during much of this period and regarded only as a supplement to grazing. A report by Graves *et al.* (5) appears to have been one of the first direct comparisons of soiling and grazing the same crop. Their data indicated a somewhat greater yield of TDN for dairy cows from grazing. At about the same time, Tretsven (21) observed no material reduction in acreage requirement, by using a combination of soiling crops when good irrigated pasture was available.

Numerous studies of the relative value of these utilization systems recently have been reported. Ittner, Lofgreen, and Meyer (11) and Meyer, Lofgreen, and Ittner (14) have reported similar gains per steer under strip and rotational grazing of alfalfa, but markedly higher beef yields per acre from strip grazing. Gullickson and Wilcox (6), Henderson, Cobble, and Cook (8), and Hale *et al.* (7) also have reported marked increases in milk or meat per acre from soiling, when compared to rotational or strip grazing, production per animal being essentially the same. However, Richard *et al.* (19) observed a greater gain per steer, as well as more efficient forage utilization from soiling as compared to strip or rotational grazing.

Experiments in which neither milk production per acre nor per cow were significantly different between utilization methods also have been reported during this period [Brundage *et al.* (2), Gullickson and Wilcox (6), Smith and Keyes (20), McMeekan (13), and Porter and Skaggs (16)].

These seemingly contrary results seem to indicate that a factor(s) other than utilization systems has been operative. The differences can not be entirely reconciled on the basis of crop species, since some species, notably alfalfa and smooth bromegrass, are common to both types of results. On the other hand, it seems quite possible that differences in stocking rate may have confounded main effects to some extent, since, in the work cited, except that of Richard *et al.* (19), higher yields per acre have been associated with higher stocking rate rather than with higher yields per animal. Ivins, Dilnot, and Davison (12) have pointed out that the portion of total pasture forage consumed in excess of the requirements to meet the potential productive ability of grazing animals will result in luxury consumption which is not reflected in additional production. McMeekan (13) has also concluded that of the major factors affecting efficiency of pasture conversion on a per-acre basis, stocking rate is by far the most powerful weapon. Thus, differential stocking rates may represent unrecognized treatment differences.

This experiment was initiated to determine the relative merits of rotational grazing, strip grazing, and soiling for milking herds being fed from improved pastures typical of the Beltsville area. These systems were compared on the following basis: (a) effect on the total nutrients utilized per acre; (b) effect on the production of milking cows; (c) effect on the relative needs of the cows for grain supplements; (d) effect on the grass-legume ratio in the sod.

EXPERIMENTAL PROCEDURE

The experimental area consisted of 12 one-acre plots on which an orchardgrass-Ladino clover pasture had been established in 1953. The utilization systems were randomly assigned to the three plots within each of four blocks. Additions of commercial fertilizer were uniform among treatments, but variable from year to year, depending on the apparent requirements of the sod (Table 1). Manure was applied to the soiling plots to compensate for the lack of manure from grazing animals.

The four plots available to each treatment were used rotationally. The grazing groups were moved from plot to plot concurrently, at intervals of from five to ten days; whereas, soiling harvests were scheduled independently, depending on the availability of sufficient forage for mechanical harvesting. Forage in excess of the needs of experimental animals was removed as hay or silage and credited to the proper system, according to its estimated TDN content (15). Thus, the number of times each plot was grazed or harvested depended on its relative position in the rotation and whether it was soiled or grazed. Each plot was managed in a way thought to be most advantageous to its particular system, and all were grazed or harvested four or five times each year.

The forage available on each plot previous to grazing or soiling was estimated by cutting sample strips of forage by the method outlined by Wagner *et al.* (22). The available dry matter was also expressed as estimated TDN, to facilitate comparison with the TDN obtained. The dried strip samples were used for estimating the percentage botanical composition of the crop.

Portable electric fences were moved daily in conjunction with the strip grazing treatment. Such fences were also used when necessary to protect parts of the rotationally grazed plots for mechanical harvesting. Improvement of fencing materials developed during the experiment have previously been reported (4)

The value of these plots for supporting milking dairy cows was measured by the TDN requirements of the cows which utilized them. Four milking cows were assigned to each system. Cows were chosen from a group that had calved 2 to 4 mo.

TABLE 1
Fertilizer applications

Application date	Method and areas	Fertilizer type	Pounds per acre
April, 1953	Band seeding, all plots	0–14–14	500
October, 1953	Broadcast, all plots	0–14–14	500
March, 1954	Broadcast, all plots	0–10–20	300
October, 1954	Broadcast, all plots	0–10–20	300
February, 1955	Broadcast, soiling plots	Manure	10,000
March, 1955	Broadcast, all plots	0–10–20	300
June, 1955	Broadcast, all plots	33–0–0	200
October, 1955	Band seeding, all plots	0–20–0	300
February, 1956	Broadcast, soiling plots	Manure	10,000
March, 1956	Broadcast, all plots	0–0–50	240
May-August, 1956	Broadcast, all plots in three applications	33–0–0	390

previously, so that animals usually remained on their assigned system throughout the season. Cows were assigned to treatments at random, from outcome groups of similar production level, live weight, breeding date, etc.

Supplemental feeding was generally at a rather low rate, with some year-to-year differences. In 1954, one Holstein and one Jersey cow under each management treatment received no grain supplement; whereas, the other Holstein and Jersey in each group received 1 lb. of grain per day for each 2 lb. of FCM produced above 24 lb. per day. During 1955 and 1956, all cows received 2 lb. of grain per day, regardless of milk production. The grain mixture used contained 16% crude protein. Hay was fed only during the first week or two of the pasture seasons.

TDN required by experimental animals was determined by the method recommended by the Joint Pasture Committee (1). The milk produced was weighed at each milking. A composite of aliquot samples of milk from four consecutive milkings was tested every ten days for butterfat content by the Babcock method. All feeds fed and refused (excepting grazed forage) were weighed. Dry matter content of the forage fed and refused for the soiling treatment was determined daily, whereas that for supplemental feeds was usually determined each ten days. The TDN content of the supplemental feeds was based on Morrison's estimates of the digestibility of similar feeds (15).

All grazing cows were on pasture continuously with the exception of two, 2-hr. periods each day, when they were brought to the barn for water, supplements, if any, and milking. Cows assigned to the soiling treatment were housed in a stanchion barn and individually fed green forage to the limits of appetite twice daily. The green forage was harvested daily with a flail type harvester and a trailer especially equipped to reduce field losses of harvested material.

When experimental plots failed to produce sufficient forage to support the cows assigned to them, the cows were continued on the same feeding system by utilizing similar nonexperimental forages. Occasionally, however, no suitable forage was available and the feeding systems were suspended for short periods. The continuity of the feeding systems is shown in Table 2.

TABLE 2

Dates of experimental and nonexperimental management of cows

Year	Management	Dates first grazing or harvest	Dates last grazing or harvest	Periods of nonexperimental management
1954	Strip grazing	April 15	September 30	Aug. 5–19[a]
	Rotational grazing	April 15	September 30	Aug. 5–19[a]
	Soiling	April 23	September 28	Aug. 5–19[a]
1955	Strip grazing	April 18	October 3	Aug. 16–31[b]
	Rotational grazing	April 18	October 3	Aug. 16–31[b]
	Soiling	April 18	October 3	Aug. 16–31[b]
1956	Strip grazing	April 25	September 18	June 21–July 3[c]
	Rotational grazing	April 25	September 16	June 21–July 3[c]
	Soiling	April 30	September 14	None

[a] All cows barn-fed during this period.
[b] All cows fed hay and permanent pasture during this period.
[c] All grazing cows fed from permanent pasture as a single group during this period.

OBSERVATIONS AND RESULTS

The productivity of all plots was seriously reduced by the abnormal rainfall pattern throughout the 3-yr. period. Very dry periods occurred in each of the pasture seasons. The only year in which the total rainfall was equal to the 26-yr. average was 1955. Even in 1955, however, two very dry periods occurred, with 14.5 in. of the yearly total occurring during an 11-day period in August. The mid-summer drought of 1954 was believed to be largely responsible for the marked reduction in Ladino clover population in all plots. This loss of clover necessitated the addition of nitrogen fertilizer in June, 1955 (Table 1). Band seeding in the sod in October, 1955, failed to re-establish the clover. The initial legume content of about 35% in the spring of 1954 was reduced to about 3% under all treatments by the spring of 1955, and remained at a low level for the remainder of the experiment. Conversely, the grass content increased from about 63 to about 95% and remained at a high level. Weed content increased slightly during this period, but was never a very large part of the spring herbage. The changes observed in botanical composition did not appear to be associated with utilization systems but with the generally dry seasons.

Estimates of the dry matter available from experimental plots have been summarized in Table 3. The amounts of dry matter available to the two grazing groups were remarkably similar throughout the experiment. Significantly greater amounts of dry matter were available to the soiling system. This greater amount of available dry matter may be explained as follows: All forage samples were cut at a uniform height (2 in.), which was characteristic of the mower. Utilization of the crop to a height of less than 2 in. (which could occur under some grazing conditions) would increase utilization of the present growth and reduce the amount available (taller than 2 in.) that would occur as regrowth for the subsequent grazing. On the other hand, leaving a stubble taller than 2 in. (which could occur when mechanically harvested) would reduce utilization of the present growth, and some growth could appear as available at more than one sampling date. Thus, these values represent the sum of amounts available as estimated at a uniform height at intervals through the season, but do not necessarily represent the total amounts produced above this height.

A summary of the utilization of forage also is presented in Table 3. The two grazing systems furnished essentially the same number of cow-days of grazing per acre. The seasonal carrying capacity of the soiling areas was consistently lower than either of the grazed areas. This was particularly true during periods when relatively small amounts of forage were available.

The amount of TDN utilized per acre by animals on the soiling system was significantly less than that utilized by either grazing treatment (Table 3). This relationship is in accord with that observed on carrying capacity. TDN harvested as excess forage was not significantly different among the treatments. The greater amounts of available forage in 1955 were reflected in greater amounts of excess forage, whereas amounts grazed remained relatively constant each year.

The amount of TDN obtained from soiling or grazing, and the amount harvested as excess, have been totaled to obtain an over-all expression of the per-acre

TABLE 3

Availability and utilization of forage under three utilization systems (per-acre basis)

Year and system	Forage available		Cow-days on experiment	Forage utilized (TDN)				TDN grazed or soiled cow-day	Total standard cow-days[d]
						Total			
	D.M.[a]	TDN[b]		Soiled or grazed	Excess[b] harvested	Lbs.	Relative rotational		
	(lb.)			*(lb.)*			*(%)*	*(lb.)*	
1954									
Rotational	3,917	2,711	133	2,261	378	2,644	100	17.0	165
Strip	4,020	2,782	133	1,925	660	2,585	98	14.5	162
Soiling	4,540	3,142	95	1,566	452[c]	2,018	76	16.5	126
1955									
Rotational	4,744	3,283	135	1,906	1,636	3,542	100	14.1	221
Strip	4,615	3,194	137	1,918	1,768	3,686	104	14.0	230
Soiling	5,426	3,755	114	1,547	1,602[c]	3,149	89	13.6	197
1956									
Rotational	3,820	2,643	132	2,198	242	2,440	100	16.7	153
Strip	3,850	2,664	134	2,284	287	2,571	105	17.0	161
Soiling	4,461	3,087	101	1,592	534[c]	2,126	87	15.8	133
3-yr. average									
Rotational	4,160	2,879	133	2,123	752	2,875	100	16.0	180
Strip	4,162	2,880	135	2,042	905	2,947	103	15.1	184
Soiling	4,809[e]	3,328	103[f]	1,568[g]	863[e]	2,431[f]	85	15.2	152

[a] Estimated from sample strips cut previously to grazing or harvest (see second paragraph under Observations and Results).
[b] Calculated from dry matter values at 69.2% TDN, Morrison's *Feeds and Feeding*, 21st ed. (clover-mixed grass hay, green).
[c] Includes credit to soiling treatment for amount harvested in excess of daily consumption.
[d] Calculated at 16 lb. TDN per standard cow-day.
[e] Significantly more than either grazing system at 1% level of probability.
[f] Significantly less than either grazing system at 1% level of probability.
[g] Significantly less than either grazing system at 5% level of probability.

productivity of each system. The lower productivity of the soiling treatment thus expressed is highly significant.

The differences between systems in calculated TDN obtained per animal per day were not significantly different, thus indicating that animals were on a fairly equal plane of nutrition. The general interpretation of the data in Table 3 made by the authors has been that cows on all systems were fed equally well while they were on the respective areas, but that soiling cows utilized less of the available forage. Hence, the carrying capacity was least.

Performance and requirements of cows while on experimental plots have been summarized in Table 4. These data substantiate the fact that the differences in yields per acre (cow-days or TDN) obtained from the utilization methods (Table 3) were not a function of differences in productivity of cow groups, since the daily requirements of cows were quite similar.

The effect of the forage utilization systems upon the cows was observed almost continuously throughout the season, in spite of the rather intermittent utilization of the tester plots (especially the soiling plots). This was accomplished by using nonexperimental areas as a forage source. Single periods of equal length representing the major portion of the season were selected for each treatment as measures of long-term effects of the systems on animal production. These data are presented in Table 5.

Some differences in average initial FCM production resulted from the random assignment of relatively few cows to a relative large number of treatments. This was especially true in 1954, when the number of treatments was doubled by using two grain-feeding levels.

The average observed milk production values per cow per season for utilization systems and years are presented in Table 5. The same values adjusted by covariance for differences in initial production are also presented.

Average values of FCM per cow per day were used in the analysis of covariance because of differing lengths of seasons. A summary of this analysis is presented in Table 7. No statistical significance could be attached to differences between methods of pasture utilization or years or to the year by utilization method interaction. Highly significant differences between years existed before adjustment for initial production level. Differences between utilization systems were not affected by the year-to-year differences in initial production levels.

Average live weight changes over the entire season also are presented in Table 5. A statistical analysis of the average live weight change per day for individual cows failed to show any significant differences due to treatment. Thus, the treatment differences in both milk production and live weight changes could not be safely considered as more than random variability.

Average seasonal milk production values for 1954 under two supplementation regimes are presented in Table 6. Although the average production of cows receiving grain was higher under all systems than that of the nonsupplemented cows, statistical analysis of the data shows these differences to be nonsignificant, and no statistically significant interaction of utilization systems and supplementation existed (Table 7). Cow variability and small numbers (two cows repre-

TABLE 4

Average performance and TDN requirements per cow per day (while on experimental plots)

Year and treatment	Days on experimental area	Performance			TDN requirements			TDN in supplements	Net TDN from pasture
		Live weight maintained	Live weight change	FCM produced	Maintenance	Live weight change	Milk		
		(lb.)							
1954									
Rotational	133	1,114	+.32	39.62	8.7	1.1	12.7	5.5	17.0
Strip	133	1,063	−.41	36.06	8.4	−1.1	11.5	4.3	14 5
Soiling	95	1,112	+.09	33.67	8.7	0.3	10.8	3.3	16.5
1955									
Rotational	135	992	+.19	22.32	7.8	0.7	7.1	1.5	14.1
Strip	137	994	+.31	20.24	7.9	1.1	6.5	1.5	14.0
Soiling	114	940	−.03	23.87	7.6	−0.1	7.6	1.5	13.6
1956									
Rotational	132	897	+.60	27.47	7.2	2.1	8.8	1.4	16.7
Strip	134	962	+.80	24.94	7.6	2.8	8.0	1.4	17.0
Soiling	101	975	+.37	25.59	7.8	1.3	8.2	1.5	15.8
Av. 1954-56									
Rotational	133	1,001	+.37	29.89	8.0	1.3	9.5	2.8	16.0
Strip	135	1,006	+.23	27.08	8.0	0.8	8.7	2.4	15.1
Soiling	103	1,009	+.14	27.71	7.9	0.5	8.9	2.1	15.2

TABLE 5
Effect of forage utilization system on animal production (entire season)

Period		Average FCM production: Initial daily level	Season total	Adjusted season total	Adjusted per cow per day[d]	Average live weight: Initial	Seasonal change
		(lb.)					
1954 (160-day)[a]							
4/15–9/21	Rotational	61.1	6,292	5,314	33.2	1,117	+54
4/15–9/21	Strip	52.6	5,518	5,067	31.7	1,148	−69
4/23–9/29	Soiling	47.1	4,913	4,800	30.0	1,117	+14
1955 (168-day)							
4/18–10/3	Rotational[b]	39.9	3,576	3,933	23.4	1,013	+31
	Strip	48.4	3,190	2,990	17.8	1,002	+52
	Soiling	42.9	3,730	3,888	23.1	933	− 5
1956 (139-day)[c]							
4/30–9/15	Rotational	39.0	3,738	4,078	29.3	844	+86
	Strip	38.6	3,397	3,761	27.1	915	+117
	Soiling	38.2	3,341	3,727	26.8	945	+52
Average							
	Rotational	46.7	4,535	4,442	28.6	991	+57
	Strip	46.5	4,035	3,939	25.5	1,022	+33
	Soiling	42.7	3,995	4,138	26.6	998	+20

[a] Includes production for August 5–19, when all cows were on barn feed.
[b] Includes production for August 16–31, when all animals were on pasture and hay.
[c] Treatments continuous except June 21–July 3, when all grazing animals were on non-experimental pasture as a single group.
[d] Adjusted for differences in initial level, analysis of covariance (Table 7).

sented in each average) resulted in low precision for detecting even rather large differences. Since any true differences or interactions appeared too small to be detected by the precision of the experiment, this variable was omitted in the two subsequent seasons.

DISCUSSION

A major advantage that might be expected from soiling is the elimination of grazing wastes. However, whether this is accomplished with a resulting

TABLE 6
Effect of forage utilization system and grain supplementation on production, 1954, pasture seasons

Treatment	Daily FCM production: Initial level	Observed average	Adjusted average[a]
	(lb.)		
With grain supplement			
Rotational grazing	64.2	45.9	38.5
Strip grazing	55.8	37.3	33.2
Soiling	48.2	33.2	32.1
Average	56.0	38.8	34.6
No grain supplement			
Rotational grazing	58.0	32.8	27.9
Strip grazing	49.4	31.7	30.1
Soiling	46.0	28.2	27.9
Average	51.1	30.9	28.6

[a] Adjusted for differences in initial level, 3-yr. combined analysis of covariance (Table 7).

TABLE 7
Analysis of variance of daily FCM with adjustments for initial level of production by covariance (mean squares only)

Source of variation	d.f.	Mean square	F.	
Years (Y)	2	92.06	2.36	N.S.[a]
Treatments (T)	2	4.28	0.11	N.S.
Y T	4	31.64	0.81	N.S.
Grain levels (G) (1954)	1	104.96	2.69	N.S.
G T (1954)	2	16.82	0.43	N.S.
Cows (C: YTG) Cows C: YTG)		23	39.06	

[a] N.S.—Indicates not statistically significant.

net gain in utilization efficiency depends upon the relative magnitude of the grazing and harvesting wastes. Therefore, if grazing wastes are sufficiently reduced and harvesting wastes remain constant, it becomes impossible to improve utilization efficiency by harvesting.

The higher per-acre production resulting from grazing, as compared to soiling, is viewed as a result of a less wasteful method of utilization, since no more forage was available to the grazing groups and performance per animal per day was essentially the same for all treatments. The magnitude of grazing wastes is closely associated with the type of crop under consideration. Relatively tall-growing and/or mature crops are much more subject to the wastes of trampling and selective grazing than are shorter and/or less mature crops. However, the wastes of soiling the taller crops will be relatively small, since stubble represents a smaller portion of the total crop and selectivity is greatly reduced.

The results and the observations made demonstrated that pasture forage, typical of this area, may be efficiently utilized by well-managed grazing. Wastes from grazing this type of pasture, however, may be considerable, particularly in the flush period of growth if understocking is allowed, or if grazing is delayed until a hay stage of maturity has been reached. It is considered important that in this experiment these difficulties were overcome by varying the area grazed per cow with portable fences and by mechanical harvesting of the forage, which was in excess of grazing requirements and approaching a mature stage.

The similar utilization efficiency of grazing systems is explained on the basis of similar amounts of wasted forage, since amounts initially available were essentially the same. The mere existence of a rotational grazing or strip grazing system has little bearing on the extent of grazing wastes, since overstocking or understocking is not necessarily eliminated by either. It is true that wastes from understocking are less likely to occur in strip grazing, since estimates of the area required can be made more easily on a daily basis than on an eight- to ten-day basis, but the possibility of equal accuracy in estimates is not necessarily precluded.

It is also recognized that strip grazing is likely to reduce the large wastes that result from rotational grazing of crops taller and/or more mature than those used in this experiment. The data indicate, however, that under certain conditions equal efficiency was attained under both systems, with no significant differences in animal response.

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SOME FACTORS AFFECTING WEIGHT GAINS OF DAIRY HEIFERS FED ALL-ROUGHAGE RATIONS[1]

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SUMMARY

Factors affecting weight gains by dairy heifers were studied with 28 heifers on pasture and ten individually fed heifers on either an all hay or grass silage ration. The heifers averaged about 450 lb. in weight. The only factor significantly correlated with gains on pasture was forage dry matter digestibility (r=.797). On pasture, the multiple correlation between average daily gains and dry matter digestibility, dry matter intake, and body weight was .831. Over a 56-day feeding period, the correlations between dry matter intake and average daily gains were .711 for silage and .399 for hay. Correlations between body weight and dry matter intake were .932 for silage and .857 for hay. Multiple correlations between average daily gains, body weight, and dry matter intake were .858 for silage and .463 for hay. Respective average daily gains for pasture, silage, and hay were 1.79, .92, and 1.34 lb. The error of estimate of weight gains in all groups prevents accurate estimate of response from the factors measured and emphasizes the large number of animals required to show significant differences between rations. On an individually fed hay ration, 82, 14, and four animals per treatment were required to show significant differences in average daily gains of .10, .25, and .50 lb. between group means. On silage, 174, 28, and seven animals were required to show the above differences. When the effects of body weight and dry matter intake of silage were removed, the number of animals needed was reduced to 72, 12, and three. The data would appear to indicate that intake was a major factor only on the grass silage.

Live weight gain is the most frequently used measure of the nutritional value of forages for dairy heifers. The adequacy of a feed for growing heifers is measured by the observed body weight gain in relation to a desired rate of gain represented by feeding standards or personal preferences. The general problems of feeding trials with dairy heifers were reviewed by Bailey *et al.* (1) and need not be repeated here. Since it is impossible to feed all forages and all combinations of forage in feeding trials, future work should emphasize principles of forage utilization and experimental efficiency.

EXPERIMENTAL PROCEDURE

The animals used in all trials were Holstein and Guernsey heifers from the station herd. The hay was a good-quality, second-cutting alfalfa, the silage was made from winter oats cut at the pre-bloom stage and ensiled without preservative, and the pasture was a winter temporary pasture consisting largely of oats and rye-grass. The hay was chopped fine and fed twice daily in quantities large enough to insure weighbacks. The silage was fed fresh and was also fed twice daily with weighbacks. All animals were individually fed and ten animals were used per treatment. The 27 pasture observations were taken from an experiment designed to study season-long changes in pasture quality. The data used were

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from animals which had been on the pasture for a minimum of 6 wk. Intake and digestibility data were determined by the chromogen–chromic oxide technique. Chromic oxide was administered twice daily in capsules, grab samples were collected twice daily and composited for each animal over a five-day collection period. The animals were fed each ration for seven days before the start of the experimental period to allow for adjustment in fill. All animals were weighed in the early afternoon; the animals on hay and silage were weighed at seven-day intervals and the pasture group at 14-day intervals.

Chromogen was determined by the technique of Reid *et al.* (5), chromic oxide by the technique of Schurch (6), and the statistical techniques were those of Snedecor (7).

RESULTS AND DISCUSSION

Factors affecting animal gains. The mean intake and performance data for the three roughages are shown in Table 1 and the relationship between the variables in Figure 1.

TABLE 1

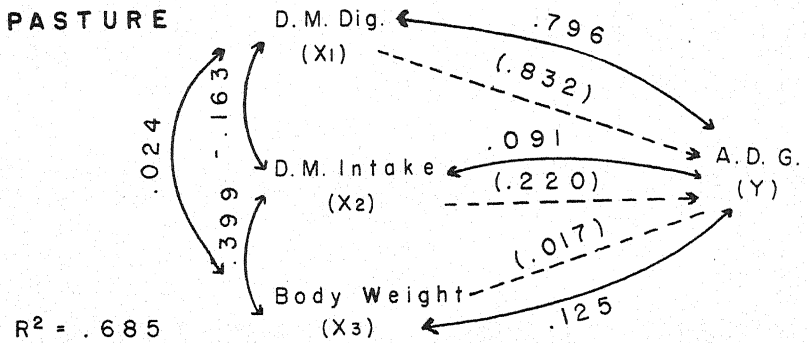
Average body weight, dry matter intake, and gain of heifers consuming the various forages

Type of forage	Range in body weight	Mean body weight	Mean dry matter intake	Correlation with body size	Mean daily gain
	(lb.)			*(r)*	*(lb.)*
Pasture	322–662	486	20.22	.399[b]	1.79
Silage	266–622	442	7.40	.932[a]	0.96
Hay	305–685	469	12.08	.897[a]	1.34

[a] Significant at 1% level of probability.
[b] Significant at 5% level of probability.

Body weight. Body weight was highly correlated with dry matter intake for the animals on the rations of hay ($r = .897$) and silage ($r = .932$). An increase in body weight of 100 lb. resulted in an increased dry matter intake of 1.39 lb. of silage or 1.36 lb. of hay. The failure of increasing body weight to show a significant increase in intake for animals on pasture may reflect either the effects of selective grazing or a general tendency for the animals to consume more than adequate quantities of forage on high-quality pasture. This relationship is in accord with the findings of Garrigus and Rusk (3) and Crampton and Purdy (2), but not with those of Martin *et al.* (4), who found an average increase in dry matter intake of 3.5 lb. for each 100-lb. increase in body weight. In no case was body weight significantly correlated with average daily gain, although with silage there was an indication that the larger animals enjoyed an advantage, since body weight accounted for 27% of the differences in average daily gain.

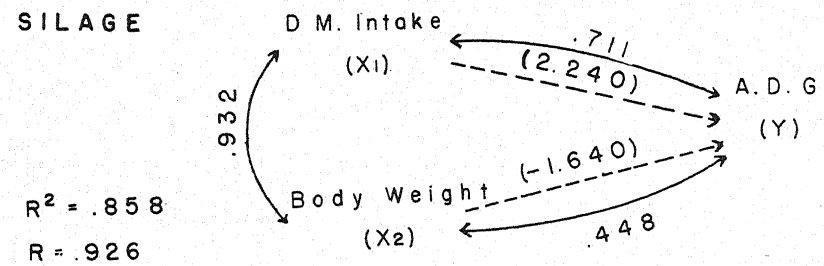
Dry matter intake. Dry matter intake was highly correlated with average daily gains only in the case of the silage, where it accounted for 58% of the variation in gains. It was apparent that the forages presented three different intake conditions. Tests for significance indicated a significant difference between forages for dry matter intake, even after adjustment for differences in average weight of animals. Silage constituted a ration on which it was difficult



$R^2 = .685$

$R = .828$

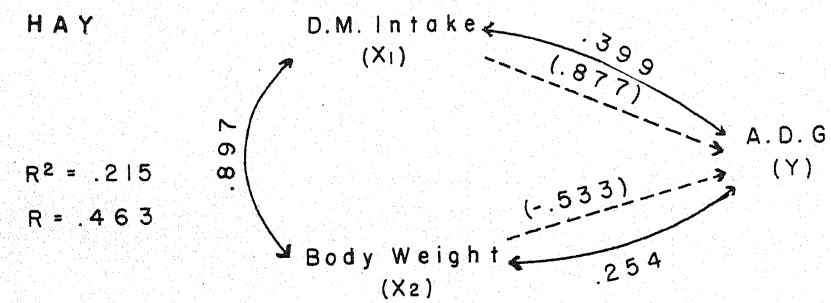
$Y = -7.70 + .1196 X_1 + .0499 X_2 + .0003 X_3$



$R^2 = .858$

$R = .926$

$Y = -.571 + .585 X_1 - .006 X_2$



$R^2 = .215$

$R = .463$

$Y = .3944 + .1089 X_1 - .0011 X_2$

FIG. 1. Factors affecting average daily gain on three types of roughage. (Path coefficients in parentheses.)

for the animals to meet their nutritive requirements, thus giving an advantage to both the larger animals and those with a greater appetite or capacity for the feed. The high-quality pasture apparently represented a forage condition conducive to large intake by even the smaller animals and adequate intakes by those with the smaller appetite or capacity. The hay ration was apparently intermediate between the two.

Differences in average daily gain due to changing forage quality were measured only in the case of the pasture. Dry matter digestibility was highly correlated with average daily gain ($r = .796$) and accounted for 60% of the variation in gains. The data indicated that forage quality is the major factor in determining the adequacy of all-forage rations for dairy heifers. On high-quality forage even small animals are able to meet their nutrient requirements, but on low-quality forages only the larger and older animals have the capacity or appetite necessary to meet their needs. It should be pointed out that further work may result in the finding of factors other than quality as measured by stage of maturity and digestibility which affect the intake of silage. If so, then silage quality must include the effects of ensiling as well as conventional quality measurements.

Duration of trial and animals required for forage evaluation. The data permitted calculations on the effect of length of feeding trials on the reduction in the standard error of average daily gain and an estimation of the number of animals required for evaluating forages of varying magnitudes in quality. These data are shown in Tables 2 and 3. Since all animals were fed the experimental ration for seven days before the start of each experiment, the seven-day weight given in Table 2 was actually made 14 days after the animal was started on the

TABLE 2

Effect of length of trial on standard error of average daily gain

Type of forage	Days on experiment							
	7	14	21	28	35	42	49	56
Pasture	....	.23	....	.24	....	.17	....	.12
Silage	.40	.30	.25	.19	.16	.14	.14	.13
Silage	(Omitting first 21 days)			.41	.22	.14	.15	.13
Hay	.50	.41	.23	.24	.15	.14	.11	.14
Hay	(Omitting first 21 days)			.39	.15	.12	.10	.10

TABLE 3

Estimated number of animals required to evaluate rations

Difference in treatment means	Number of animals required		
	Hay	Silage	
		Effects not removed[a]	Effects removed
(lb.)			
0.10	82	174	72
0.25	14	28	12
0.50	4	7	3

[a] "Effects" refers to body weight and dry matter intake. There was no advantage in this correction on the hay group.

feed. Data in Table 2 indicate that a satisfactory standard error required a 56-day experimental period, although the reduction in standard error was slight after 42 days. A comparison of the data for silage and hay, calculated by using the 21-day weight as the starting point (actually 28 days after the animal was started on feed), indicates that the standard errors are composed of at least two factors. The standard error is reduced by the animal becoming accustomed to the ration and also by a sufficient length of time to overcome the magnification of errors due to small differences in body weight between weigh periods. The latter factor probably operates largely through weighing errors due to small changes in fill which are important only when small differences in body weights are being considered. A further contributing factor may be differences between animals in the acceptance of the rations. This may explain, in part, the seemingly greater accuracy of early weighings of the animals on pasture, since their average daily gain was greater and the weigh periods were at 14- rather than seven-day intervals.

Estimates of the number of animals required to determine significant differences in treatment means of 0.10, 0.25, and 0.50 lb. average daily gain are shown in Table 3. The large numbers of animals required for 0.10 lb. reflect the large standard error in average daily gains and the many factors (largely unknown) which govern the growth of animals. The large reduction in animal numbers resulting from removing the effects of body weight and individual intakes on weight gain in the case of silage indicates the importance of knowing and removing factors affecting weight gain, other than those due to ration, whenever possible. The data also indicate that small differences in forage quality can not be measured with the animal numbers usually available for experimental purposes. Similar data for pasture experiments were not calculated, since pastures vary in quantity as well as quality of forage, although animal numbers similar to those given for hay should be expected when great care is exercised to keep only forage quality as a variable.

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NUTRIENT LOSSES, QUALITY, AND FEEDING VALUES OF WILTED AND DIRECT-CUT ORCHARDGRASS STORED IN BUNKER AND TOWER SILOS

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SUMMARY

First-cutting orchardgrass was preserved as: 1. Wilted silage stored in a tower silo. 2. Direct-cut, sodium metabisulfite–treated silage, stored in a tower silo. 3. Direct-cut sodium metabisulfite–treated silage, stored in a bunker silo. These silages were prepared in each of two successive years and all silages were sealed with weighted plastic covers. The silages were compared on the basis of storage losses, storage temperatures, chemical quality, feeding trials, and digestion trials. An expression of silage feeding value which combines dry matter intake rate and TDN content has been proposed.

Tower storage of wilted forage and bunker storage of the direct-cut forage were about equally efficient in preserving feed nutrients. Tower storage of direct-cut forage was consistently less efficient, primarily because of higher seepage losses. All silages were of good chemical quality and of about equally high feeding value. The wilted silage, however, contained more butyric acid in both years and was consumed by dairy cattle in significantly greater amounts one year.

The bunker silo, as used in this experiment, was a more suitable storage structure for direct-cut orchardgrass than a tower silo. However, when forages were stored in tower silos, the wilting method was generally more satisfactory than direct-cutting combined with sodium metabisulfite treatment.

Difficulties in obtaining well-preserved and palatable silages from direct-cut, high-moisture hay crops have been repeatedly although not consistently observed. Mitigation of these problems by wilting the crop, as demonstrated by Woodward and Shepherd (11), has been reported by the Vermont and Ohio workers (8, 9), as well as by numerous other investigators. Wilting is now one of the generally accepted ensiling procedures. Improved preservation and/or increased palatability by the use of sodium metabisulfite applied to the unwilted silage has also proven beneficial under some conditions (1, 2, 5). Although both of these procedures have produced some beneficial effects, few direct comparisons between the two methods have been reported. A direct comparison of this type, reported by Cowan *et al.* (2), indicated a marked advantage of bisulfite treatment over wilting for the dry matter preservation of pre-bloom and full-bloom alfalfa, but no advantage for 1/10-bloom alfalfa. However, these results may not provide a practical comparison of methods, since the silages were made in 4- by 8-ft. silos, and dry matter losses of up to 35% were observed in the wilted silage. Other investigations (10, 11), using farm-sized silos, have reported losses of about one-third this amount from wilted silage.

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The relative value of bunker silos and tower silos can not be accurately assessed from previously published reports. Cowan *et al.* (3) and Gordon *et al.* (4) have reported quantitative evaluations of trench and bunker silos, respectively, but no direct comparisons with tower silos were included in these experiments. A critical comparison of these two silo types demands concurrent use with the same crop. This experiment was, therefore, designed to allow the following direct comparisons: 1. Wilting vs. sodium metabisulfite treatment of an unwilted crop, both stored in tower silos. 2. Tower vs. bunker storage, when both silages were unwilted, treated with sodium metabisulfite, and sealed by the best practical methods available.

EXPERIMENTAL PROCEDURES

The crops used in this investigation were harvested in 1956 (Experiment 1) and 1957 (Experiment 2), and fed during the winters following each harvest.

Experiment 1. Forages from a first-cutting crop of orchardgrass were harvested and stored concurrently by three different methods. All forages were chopped with a forage harvester set for a 3/8-in. cut. The procedures in harvesting and storing the forage were as follows: 1. Field-wilted to an average moisture content of about 70%, stored with no additive in a 10- by 25-ft. concrete tower silo. 2. Not wilted, stored in a 10- by 25-ft. concrete tower silo with 8 lb. of sodium metabisulfite (SMB) per ton of forage added at the blower. The tower silos were alternately leveled and tramped during the filling period. 3. Not wilted and stored in a 15- by 66- by 8-ft. drive-through bunker silo. Sodium metabisulfite (SMB) at the rate of 8 lb. per ton of forage was manually applied to each load of forage after it had been spread in the bunker. Bunker-stored forage was packed with a wheel-type tractor during the filling period.

Filling was started on May 2, when most of the grass heads were visible but not fully emerged from the boot. Rain interrupted the filling operation after two loads of direct-cut forage had been stored in the tower silo. A temporary plastic seal prevented spoilage in this silo until filling was resumed. Filling of all structures was completed during the May 7-11 period.

Bunker-stored silage was sealed May 11 with a neoprene-nylon blanket weighted with a 2-3 in. layer of sawdust. Tower-stored silages were sealed with sheets of polyethylene weighted with nonexperimental forage. Guttering and flow meters were utilized to measure the seepage from each silo.

Each load of stored forage was weighed and sampled for chemical analyses. Silage temperatures during storage in tower silos were measured by thermocouples located at 0.5, 6.5, 12.5, 18.5, and 24.5 ft. above the silo floor. Silage removed for feeding was weighed and sampled daily. Five-day composite samples were analyzed for dry matter (toluene distillation) pH, sugar, carotene, crude protein, ammoniacal nitrogen, and organic acids. Dried 20-day composites were analyzed for the remaining constituents.

The silos were opened and feeding commenced after a storage period of about 6 mo. Feeding values of the silages were estimated with 12 milking cows in a 120-day 3×3 Latin-square feeding trial. Each period consisted of a ten-day change-over and 30-day experimental period.

The rations consisted of the experimental silage, fed to the extent of appetite, and grain fed according to the equalized feeding system described by Lucas (6). The grain ration, composed of cornmeal 44%, ground oats 22%, wheat bran 22%, soybean oil meal 11%, and salt 1%, was fed at a grain : FCM ratio of about 1 : 4.

Data were obtained on a daily individual cow basis for the weights of feeds fed and refused and milk produced. Butterfat content of the milk was determined for each cow from two-day composite samples taken once during each ten-day period. The dry matter content of refused feed was determined at five-day intervals and that of the grain each 20 days. Sampling and analysis of the silages fed have been described.

Dry matter digestibility and TDN content of the silages were determined by the total collection method after the completion of the feeding trial. The same animals and ration assignments (excepting the omission of grain) that were used during the last feeding trial period were extended for the digestion trials. Thus, a randomized block of four replications of three cows each was the design used for digestibility trials.

Experiment 2. The procedures and methods used were generally the same as those used in Experiment 1, with the following exceptions: Filling was accomplished without interruption during the period May 6–10. The tower silos had been enlarged to a height of 35 ft. Thermocouples were located at 3-ft. intervals up to 27 ft. above the silo floor. Thermocouples were also located in the middle of the bunker silo at 1.5, 3.0, 4.0, and 5.5 ft. above the floor, and at 8 ft. from the end of the bunker at 3.0 and 4.0 ft. from the floor. Four thermocouples were distributed transversely at each of the above bunker locations, making a total of 24 points in the bunker. The digestion trials were conducted concurrent with the feeding trial. Three additional cows on all silage rations were utilized for this purpose in a 3×3 Latin-square design.

RESULTS

The chemical characteristics of stored forages (Table 1) within each experiment were very similar, with the exception of a higher dry matter content in the

TABLE 1
Average chemical composition of forages as stored

Constituents[a]	1956 Crop treatments			1957 Crop treatments		
	Wilted in tower	Direct-cut in tower	Direct-cut in bunker	Wilted in tower	Direct-cut in tower	Direct-cut in bunker
Dry matter	30.0	22.2	21.8	30.8	23.8	25.2
Crude protein	12.8	13.6	13.5	13.0	13.4	13.2
Ether extract	3.1	3.4	3.2	2.9	3.3	3.3
Crude fiber	25.9	24.9	24.9	27.3	26.5	26.6
N.F.E.	49.4	47.4	47.9	48.3	46.8	47.5
Ash	8.8	10.6	10.6	8.0	10.0	9.4
Red. sugars	8.6	8.6	8.4	10.5	10.3	10.1
Carotene	163.7	199.3	194.2	205.9	226.4	214.6

[a] All constituents except dry matter and carotene expressed as per cent of dry matter. Carotene expressed as $\mu g/g$ of dry matter.

wilted forage and a higher ash content in the SMB-treated forages. Wilting apparently reduced the carotene content, particularly in Experiment 1. The most noticeable difference between years in the composition of forages was the higher dry matter content of the 1957 unwilted crops.

Average chemical composition of the silages removed for feeding is presented in Table 2. The higher dry matter content resulting from wilting persisted through the storage period, although the difference was reduced by an increase in per cent dry matter in the tower-stored unwilted silages. This increase is an expected result of the loss of moisture through seepage.

TABLE 2
Average chemical composition of silages as fed

	1956 Crop treatments			1957 Crop treatments		
Constituents[a]	Wilted in tower	Direct-cut in tower	Direct-cut in bunker	Wilted in tower	Direct-cut in tower	Direct-cut in bunker
Dry matter	29.0	25.4	22.4	31.4	27.4	25.0
Crude protein	14.4	13.5	14.2	13.0	12.5	13.0
Ether extract	3.3	3.5	3.6	3.9	3.5	3.7
Crude fiber	29.5	29.8	28.8	30.4	29.3	28.6
N.F.E.	42.7	43.1	42.5	44.7	45.8	45.5
Ash	10.2	10.1	10.9	7.9	9.0	9.2
Sugar	0.8	2.6	3.0	3.4	4.4	4.7
Carotene	215.6	275.5	236.1	179.4	241.4	215.1
pH	4.2	4.2	4.4	4.2	4.2	4.2
Ammoniacal nitrogen	12.5	10.1	9.7	11.4	8.9	7.5
Butyric Acid	2.1	0.4	0.5	1.2	0.3	0.4
Acetic Acid	1.9	1.2	1.5	1.4	1.3	1.4
Propionic Acid	0.2	0.1	0.1	0.2	0.1	0.1
Lactic Acid	5.2	4.2	3.8	5.8	5.2	5.0

[a] Ammoniacal nitrogen expressed as per cent of total nitrogen. Carotene as $\mu g/g$ of dry matter; all other constituents except dry matter and pH expressed as per cent of dry matter.

Higher total reducing sugar and carotene contents characterized the SMB silages in both years. This rather commonly observed effect is of questionable nutritional significance, although the higher sugar content may be indicative of a less intense fermentation. Protein degradation, as indicated by ammoniacal nitrogen values, was quite well controlled in all silages, although it tended to be higher in the wilted silages.

Organic acid concentrations and pH values indicated that all of the tower-stored silages were of excellent quality. However, somewhat greater amounts of organic acid were observed in the wilted than in the SMB-treated silage in both experiments. The wilted silage had a pleasant odor in spite of its higher butyric acid content. Chemical quality of the bunker-stored silage was generally equal to that obtained by tower storage, in both years.

Forage dry matter was most efficiently preserved by wilting and storage in a tower silo, and least efficiently preserved by direct cutting combined with SMB treatment and tower storage (Table 3). Bunker-storage efficiency exceeded that of tower storage for direct-cut forage and was nearly equal to that obtained by wilting and tower storage. A generally higher rate of dry matter recovery for all

TABLE 3
Percentage of stored nutrients preserved for feeding

Nutrients	1956 Crop treatments			1957 Crop treatments		
	Wilted in tower	Direct-cut in tower	Direct-cut in bunker	Wilted in tower	Direct-cut in tower	Direct-cut in bunker
Dry matter	86.3	77.9	85.2	92.1	87.0	90.3
Crude protein	96.7	77.4	89.9	92.2	81.1	89.3
Ether extract	91.7	80.6	96.3	125.4	92.1	101.2
Crude fiber	98.2	93.0	98.7	100.7	96.0	97.2
N.F.E.	74.6	70.8	75.6	85.2	85.0	86.5
Ash	99.5	73.9	87.7	91.3	78.3	87.8
Sugar	8.2	23.5	30.1	29.3	37.1	42.1
Carotene	113.6	107.7	103.6	80.2	92.7	90.5

treatments was observed in Experiment 2. Better preservation of NFE and reducing sugars in Experiment 2 contributed noticeably to this difference.

The sources of dry matter loss by treatments are shown in Table 4. Top spoilage was negligible in all of the treatments, that occurring in the bunker silos being confined almost entirely to a narrow surface band next to the side walls. Seepage losses for the direct-cut tower-stored silages were four to five times greater than in the wilted silages and two to three times greater than in the bunker-stored silages. Differences between treatments with respect to gaseous losses were neither great nor consistent from year to year. However, gaseous losses for all treatments were much lower in Experiment 2 than in Experiment 1. The gaseous losses from plant respiration and/or through microbial activity were apparently less in Experiment 2. The lower losses of NFE and reducing sugars tend to support this observation, but the reasons for lower activity of this type are not apparent.

Average temperatures of Experiment 1, tower-stored silages, are presented in Table 5. The wilted silage reached a peak temperature of 80° F. four days after filling, after which the temperature declined until higher ambient temperatures were encountered in July. The temperature of direct-cut tower-stored silage increased to 79° F. during the ten days following filling, and remained rather constant until July. The relatively low temperatures of both silages indicated that air was very effectively excluded. The good-quality silage which resulted demonstrates that the development of higher temperatures is not necessarily a requirement of good ensiling techniques.

The temperatures of Experiment 2 silages also are shown in Table 5. Temperatures in the tower silos were higher than had been observed in the previous year.

TABLE 4
Distribution of dry matter losses (per cent of stored dry matter)

Source of loss	1956 Crop treatments			1957 Crop treatments		
	Wilted in tower	Direct-cut in tower	Direct-cut in bunker	Wilted in tower	Direct-cut in tower	Direct-cut in bunker
Spoilage	0	0	0.4	0	0	2.5
Seepage	1.7	8.2	3.7	1.4	6.6	1.8
Gaseous	12.0	13.9	10.7	6.5	6.4	5.4
Total	13.7	22.1	14.8	7.9	13.0	9.7

TABLE 5
Average silage temperatures, ° F.

Experiment 1[a]				Experiment 2[b]				
		Silage treatment				Silage treatment		
Date	Days in storage	Wilted tower-stored	Direct-cut tower-stored	Date	Days in storage	Wilted tower-stored	Direct-cut tower-stored	Direct-cut bunker-stored
May 12	1	74	71	May 11	1	95	82	96
13	2	76	71	13	3	96	86	96
15	4	80	72	16	6	97	89	95
17	6	80	73	17	7	97	89	95
19	8	78	77	20	10	97	87	93
21	10	77	79	23	13	96	86	91
26	15	76	79	27	17	95	83	89
31	20	74	80	31	21	92	83	88
June 5	25	74	79	June 12	33	87	81	83
10	30	73	79	21	42	85	81	82
20	40	71	80	28	49	86	82	82
30	50	71	81	July 9	60	83	82	80
July 10	60	73	83	12	63	82	82	84
20	70	75	85	19	70	81	82	79

[a] Mean values for ½, 6½, and 12½ ft. above silo floor.
[b] Mean values for 3, 9, and 15 ft. above tower silo floors and mean values for all points in bunker silo.

Average maximum air temperatures during the filling periods were 72 and 77° F. for the first and second experiments, respectively, which could account for some of the differences in silage temperatures. Wilted silage and direct-cut tower-stored silage attained peak temperatures of 97 and 89° F., respectively, after six days of storage.

Temperatures at the several points in the bunker silo (Experiment 2) showed a range of 26° F. (81–107) one day after filling. These values gradually declined and became more uniform as the storage period progressed. Temperature values did not appear to be related to the depth of the silage, and the values in areas separated by only 1 ft. vertical distance differed by as much as 19° F. This seemingly random distribution may have been a reflection of differences in exposure to air during the filling process. Temperatures at a given location were rather uniform with respect to horizontal distribution. The average values for all points in the bunker silo on selected dates are presented in Table 5.

Results of the feeding and digestion trials are presented in Table 6. Comparisons of treatment effects within each year by analysis of variance indicated no significant differences in live weight gains or FCM production. The rates of FCM decline (regression) in Experiment 1 are deserving of special comment. Cows fed direct-cut tower-stored silage declined more rapidly in production during the preliminary periods than the two other groups. However, after their initial drop (2.26 lb. per cow per ten days) production of these animals increased somewhat during the remainder of the period (+0.96 lb. per cow per ten days). These circumstances resulted in similar average FCM production values for all treatments, in spite of the significantly different regressions during the experimental period (days 11–40).

TABLE 6

Results of feeding and digestibility trials

	Experiment 1			Experiment 2		
	Wilted tower-stored	Direct-cut tower-stored	Direct-cut bunker-stored	Wilted tower-stored	Direct-cut tower-stored	Direct-cut bunker-stored
Feed dry matter consumed *(pounds per cow per day)*						
Silage	23.34[c, d]	20.47[c]	20.06[d]	22.68	22.39	22.45
Concentrates	5.71	5.76	5.70	5.79	5.77	5.75
Total	29.05	26.23	25.76	28.47	28.16	28.10
(Pounds per 100 lb. live weight per day)						
Silage	2.34	2.09	2.07	2.05	2.04	2.07
Concentrates	0.57	0.59	0.59	0.53	.53	0.53
Total	2.91	2.67	2.66	2.57	2.56	2.59
Milk production (FCM) *(pounds per cow)*						
Average per day	24.51	23.53	25.11	24.54	25.73	25.60
10-day regression[a] (lb.)	—.35	+.96[e]	—.55[e]	—.68	—1.12	—.91
10-day regression[b] (lb.)	—1.47	—2.26	—1.09	—1.31	—.88	—.24
Ratio, Grain: Milk	1:4.3	1:4.1	1:4.4	1:4.2	1:4.5	1:4.5
Live weight per cow *(lb.)*						
Average	998	982	967	1,108	1,098	1,085
10-day regression[a]	+15.4	+15.2	+9.7	+11.2	+7.1	+13.1
Calculated feeding value						
Total daily requirement *(lb. TDN)*	21.18	21.70	19.16	20.50	19.34	21.31
Concentrate value *(lb. TDN)*	4.80	4.84	4.79	4.86	4.85	4.83
Required from silage *(lb. TDN)*	16.38	16.86	14.37	15.64	14.49	16.48
TDN content of silage, dry matter *(%)*	70.2	82.4	71.6	69.0	64.7	73.4
Determined feeding values of silages						
TDN in dry matter *(%)*	67.1	70.3	68.8	69.2	68.4	66.9
Dry matter digestibility (%)	69.0	70.9	71.6	69.6	70.1	69.0
Energy digestibility *(%)*				67.9	68.4	67.5
Feeding potential						
Feedable TDN/day *(lb.)*	15.66	14.39	13.80	15.69	15.31	15.02
Relative	113	104	100	104	102	100

[a] Regression by ten-day periods ending 20, 30, and 40 days after ration changes.

[b] Regression by ten-day periods ending ten and 20 days after ration changes.

[c, d, e] Difference in values followed by the same letter are statistically significant at the 1% level of probability.

Consumption of silage dry matter was significantly higher from wilted silage than from the two other silages in Experiment 1. However, dry matter intakes from the three silages in Experiment 2 were nearly identical.

Feeding values as determined by total collection digestion trials are presented in Table 6. All of the TDN values are above Morrison's (7) average value for orchardgrass silage, and there were no statistically significant differences among silages in either experiment. However, TDN values, calculated from estimated animal requirements, indicated that the silages varied widely in TDN content. This discrepancy between determined and calculated values indicates that ad lib. feeding will provide an expression of differences in silage dry matter intake, but is not well suited for TDN evaluations. When energy intakes are above requirements for milk production and body maintenance, the apparent energy value of a feed is profoundly affected by the relative efficiency of animals in converting feed energy to body energy and by the precision with which these changes can be measured. The efficiency of the animal also may be affected by level of feeding. Thus, silage energy evaluations by feeding trials with milking cows are at best rather crude and should be conducted on an equal dry matter intake basis over longer periods, in order to reduce these errors.

An ideal expression of feeding value should indicate the rate at which a feed will be consumed, as well as its nutritive value per unit of weight. Such an expression (pounds of silage dry matter consumed times determined per cent TDN) may be calculated from these data. This expression of feeding potential is presented in Table 6 as pounds of TDN per day and in relative terms. The relative rates of dry matter consumption are applicable only to the silages within an experiment which were directly compared under the same feeding condition; therefore, the feeding potential values are limited in the same way. The feeding potential of the Experiment 1 wilted silage was appreciably higher than the two other silages of that experiment. Other differences in feeding potential do not appear to be important.

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EFFECT OF AUREOMYCIN, ERYTHROMYCIN, AND HYGROMYCIN ON THE GROWTH RATE AND WELL-BEING OF YOUNG DAIRY CALVES[1]

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SUMMARY

Eighty-eight two-day-old calves were used in two 84-day experiments to evaluate the antibiotics aureomycin, erythromycin, and hygromycin as supplements to a milk replacer–starter type of ration. Hygromycin was fed at five levels ranging from 3.0 to 50 mg. of hygromycin per pound of replacer. A milk replacer–calf starter–hay system of feeding was used in both experiments.

Supplementing the rations with either aureomycin or erythromycin improved the rate of gain of the calves, increased the feed consumption, and reduced the incidence of calf scours. Hygromycin did not improve weight gains or skeletal growth of the calves, but did significantly reduce the incidence of calf scours. All levels of hygromycin tended to reduce the consumption of calf starter, particularly when fed at high levels.

The influence of several antibiotics, including aureomycin, on the growth and well-being of young dairy calves has been established and reviewed by Lassiter (1), but the value of feeding calves erythromycin and hygromycin has not been reported.

The purpose of this study was to evaluate the antibiotics erythromycin and hygromycin in comparison to aureomycin as constituents of calf feeds.

The results reported cover two separate experiments and will be designated as Trials I and II.

EXPERIMENTAL PROCEDURE

Trial I. Two-day-old Holstein calves were divided into five comparable groups and raised to 86 days of age on a milk replacer–starter–hay feeding program. The calves were permitted to remain with their dams for approximately 48 hr. after birth. Each calf was then placed in an individual pen, weighed, and subjected to one of five experimental regimes. The composition of the basal milk replacer follows: dried skimmilk—52.4 lb., cerelose—30 lb., corn distillers dried solubles—10 lb., dried whey—5 lb., dicalcium phosphate—2 lb., trace mineral salt—0.5 lb., and vitamin and mineral mix—0.1 lb. (3). All calves were fed the basal calf starter beginning at 1 wk. of age, composed of ground yellow corn—549 lb., crimped oats—470 lb., linseed oil meal—100 lb., soybean oil meal—100 lb., corn distillers dried solubles—150 lb., cane molasses—100 lb., dicalcium phosphate—15 lb., trace mineral salt—15 lb., and vitamin A and D supplement—1 lb. The basal milk replacer contained approximately 22% crude protein and the calf starter 16% crude protein.

Group 1 received the basal milk replacer and the basal calf starter, but no antibiotic supplement; Group 2 received the basal milk replacer supplemented with 50 mg. of aureomycin per pound and the basal calf starter supplemented

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with 15 mg. of aureomycin per pound; Group 3 received the basal milk replacer supplemented with 50 mg. of erythromycin per pound and the basal calf starter supplemented with 15 mg. per pound of erythromycin; Group 4 received the basal milk replacer supplemented with 50 mg. of hygromycin per pound and the basal calf starter supplemented with 15 mg. of hygromycin per pound; and Group 5 received the basal milk replacer supplemented with 25 mg. of hygromycin per pound and the basal calf starter supplemented with 7.5 mg. of hygromycin per pound. All calves were fed a good quality alfalfa–brome grass hay, beginning at 1 wk. of age. All milk replacers were fed according to a prearranged schedule (Table 1) which was designed to have the calves completely

TABLE 1
Milk replacer feeding schedule

Age	Milk	Milk replacer	Water
(days)		*(lb/day)*	
0 – 2	Dam		
3 – 10	4.0	0.6	4.0
11 – 14	2.0	1.0	7.0
15 – 28	....	1.4	12.0
29 – 75	....	1.0	12.0
36 – 42	....	0.6	12.0
43 – 49	....	0.4	12.0
50 – over	....	None	

off the replacer feeding at 50 days of age. All starter and hay feeding was completely ad libitum, with the exception that starter consumption was limited to 5 lb. per calf daily. Complete records were kept on total feed intake, growth, scouring, and general health of the calves. The calves were weighed weekly and measurements of the height at withers and chest circumference were made at the beginning and end of the experimental period.

RESULTS AND DISCUSSION

Results of Trial I. The effect of the three antibiotics on the daily gain of the calves is shown in Table 2, effect on skeletal growth and incidence of scours in Table 3, and on feed consumption and feed efficiency in Table 4.

TABLE 2
Effect of aureomycin, erythromycin, and two levels of hygromycin on the average daily gain of Holstein calves (Experiment I)

Group	Initial Weight	Age in weeks		
		0–4	0–7	0–12
	(lb.)		*(lb/day)*	
1—Basal	97.6	0.43	0.65	1.04
2—Basal plus aureomycin	97.5	0.49	0.84	1.21
3—Basal plus erythromycin	96.6	0.44	0.68	1.12
4—Basal plus hygromycin (50 mg/lb replacer)	97.8	0.28	0.61	0.96
5—Basal plus hygromycin (25 mg/lb replacer)	97.9	0.35	0.71	1.01
L.S.D.—1%	n.s.	n.s.	n.s.	0.12
L.S.D.—5%	n.s.	n.s.	n.s.	0.09

n.s. = Nonsignificant.

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TABLE 3

Effect of various antibiotics on skeletal growth and incidence of scours in calves from birth to 12 wk. of age (Experiment I)

Group	Average increase in		Scours
	Ht. of withers	Chest circumference	
	(in.)	*(in.)*	*(days/calf)*
1—Basal	4.62	7.00	11.0
2—Basal plus aureomycin	5.09	8.62	4.2
3—Basal plus erythromycin	4.84	8.31	3.1
4—Basal plus hygromycin (50 mg/lb replacer)	3.34	6.34	3.1
5—Basal plus hygromycin (25 mg/lb replacer)	4.59	6.84	4.2
L.S.D.—1%	0.54	n.s.	2.45
L.S.D.—5%	0.41	0.24	

n.s. = Nonsignificant.

The average initial weight of the five experimental groups was essentially the same; therefore, it should not have had any influence on the performances of the various rations (Table 2). The influence of the various antibiotics on the average daily gain of the calves at different ages is shown in Table 2. Aureomycin appeared to improve the growth rate of the calves more than did the two other antibiotics. The growth rate of the calves fed the rations containing aureomycin and erythromycin was significantly greater (2) than the growth rate of the calves fed the control ration or any of the rations containing hygromycin. Under the conditions of this experiment, and the level of supplementation employed, hygromycin did not appear to improve the growth rate of the calves. There was some indication that the growth rate of Group 5 calves was improved over that of the calves of Group 4, which might indicate that the level of supplementation with hygromycin was too high. This appears to be particularly evident as shown by the growth rate of the calves from 0 to 4 wk. of age. Both groups of hygromycin-fed calves exhibited a slower rate of growth during the first 4 wk. of life than any of the other antibiotic groups and the control calves.

TABLE 4

Effect of various antibiotics on average total feed consumption and feed efficiency of calves from birth to 12 wk. of age (Experiment I)

Group	Average total consumption				Feed/lb of gain
	Milk	Replacer	Starter	Hay	
			(lb.)		
1—Basal	40.0	42.4	188.1	43.8	3.2
2—Basal plus aureomycin	40.0	42.4	220.4	45.0	3.1
3—Basal plus erythromycin	40.0	42.4	201.7	34.8	3.0
4—Basal plus hygromycin (50 mg/lb replacer)	40.0	42.4	158.5	42.4	3.1
5—Basal plus hygromycin (25 mg/lb replacer)	40.0	42.4	162.7	53.8	3.1
L.S.D.—1%			n.s.	n.s.	n.s.
L.S.D.—5%			19.3	n.s.	n.s.

n.s. = Nonsignificant.

Skeletal growth data as shown in Table 3 show a trend similar to those of weight gains.

Under the conditions of this study, all of the antibiotics tested appeared definitely to decrease the incidence of scours or diarrhea, and all of the antibiotics appeared to be equally effective in this respect. As shown in Table 4, calves fed the ration containing aureomycin had the greatest consumption of starter and total feed. It appears also that hygromycin at both levels had a depressing effect upon the consumption of calf starter, but did not appear to have any appreciable effect on the consumption of hay. There were no significant differences among the various rations in respect to feed utilization; however, all of the antibiotics tended to increase the efficiency of utilization, but the calves fed the ration containing erythromycin had the highest efficiency.

Trial II. Forty-eight, two-day-old Holstein male and female calves were divided into six similar groups, based on sex and body weight. Group 1 received only a basal milk replacer and calf starter; Group 2 received the basal milk replacer supplemented with 50 mg. of aureomycin per pound and the basal calf starter supplemented with 15 mg. of aureomycin per pound; Groups 3, 4, 5, and 6 received the basal milk replacer supplemented with 3.0, 6.0, 12.0, and 25 mg., respectively, of hygromycin per pound and the basal calf starter supplemented with 1.0, 2.0, 4.0, and 7.5 mg., respectively, of hygromycin per pound. The basal milk replacer was composed of dried skimmilk—34.0 lb., dried whey—37.0 lb., cerelose—16.4 lb., corn distillers dried solubles—10.0 lb., trace mineral salt—0.5 lb., dicalcium phosphate—2.0 lb., and a vitamin and mineral mix—0.1 lb. The same calf starter was fed in this trial as was fed in Trial I. The basal milk replacer contained 20.7% crude protein. The feeding and management of the calves were essentially the same as outlined in Trial I.

Results of Trial II. As shown in Table 5, there were no significant differences among groups regardless of treatment, even though the differences in starting body weight were corrected by covariance analysis (2). Aureomycin-fed calves made slightly faster gains than the other groups up to 12 wk. of age and all groups of calves fed hygromycin made slightly slower gains than calves fed the basal ration. There appeared to be very little difference in the weight gains of

TABLE 5

Effect of aureomycin and four levels of hygromycin on the average daily gain of Holstein calves (Experiment II)[a]

Group	Average initial weight	Age in weeks	
		0–6	0–12
	(lb.)		
1—Basal	95.2	0.61	1.00
2—Basal plus aureomycin	99.8	0.62	1.08
Basal plus four levels of hygromycin			
3—(3 mg/lb replacer—1 mg/lb starter)	100.4	0.48	0.89
4—(6 mg/lb replacer—2 mg/lb starter)	93.0	0.48	0.89
5—(12 mg/lb replacer—4 mg/lb starter)	91.4	0.62	0.91
6—(25 mg/lb replacer—7.5 mg/lb starter)	92.9	0.49	0.89

[a] No significant differences among groups.

calves fed the four levels of hygromycin. In Trial I, both groups of calves fed hygromycin made weight gains slightly less than control calves. It would appear that the reduced rate of growth of calves fed hygromycin in Trial I was not due to an excessively high level of hygromycin.

There were no significant differences among groups in respect to increase in height at withers or chest circumference. None of the groups of calves had a very high rate of scouring and there was little evidence to indicate that either antibiotic had any significant effect. The calves averaged only 1.0, 0.6, 0.9, 1.0, 0.2, and 0.1 days per calf for Groups 1 to 6, respectively. The small differences among groups were not statistically significant.

The effect of the two antibiotics on feed consumption and feed required per pound of gain is presented in Table 6. The calves fed the ration containing aureomycin consumed slightly more starter than the control calves, or the calves fed any of the four levels of hygromycin, but the increase in starter consumption was not statistically significant. The calves fed the higher levels of hygromycin consumed less starter than any of the calves in the other groups, which confirms

TABLE 6

Effect of aureomycin and four levels of hygromycin on average total food consumption and efficiency of calves from birth to 12 wk. of age (Experiment II)

Group	Average total consumption				Feed/lb of gain
	Milk	Starter	Replacer	Hay	
	(lb.)				
1—Basal	40	42.4	218.1	69.3	4.1
2—Basal plus aureomycin	40	42.4	237.1	73.9	4.0
Basal plus four levels of hygromycin					
3—(3 mg/lb replacer—1 mg/lb starter)	40	42.4	215.2	79.0	4.7
4—(6 mg/lb replacer—2 mg/lb starter)	40	42.4	219.5	63.2	4.4
5—(12 mg/lb replacer—4 mg/lb starter)	40	42.4	212.1	75.2	4.4
6—(25 mg/lb replacer—7.5 mg/lb starter)	40	42.4	188.3	68.2	4.8

the observations made in Trial I. Very little difference existed among groups in hay consumption or feed required per pound gain.

The results of the two trials indicate that aureomycin and erythromycin improved the rate of gain in body weight of young dairy calves, reduced the incidence of calf scours, and increased the consumption of feed, particularly grain. The antibiotic hygromycin, at the levels fed in these studies, did not increase the rate of gain or feed consumption. In fact, at high levels, hygromycin depressed starter consumption, but it did appear to reduce the incidence of calf scours.

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UNUSUAL MAGNITUDE AND VARIATION OF PLASMA PROTEIN-BOUND IODINE VALUES OF DAIRY CATTLE[1,2]

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SUMMARY

Plasma PBI values of unusual magnitude and variations have been observed in the dairy herd at West Virginia University. The appearance of high PBI values is seasonal, occurring mostly in the late winter and spring. Plasma PBI follows a definite pattern with time by rising to a peak and then declining. A study of management practices revealed that the rise in PBI paralleled the feeding of grass silage to the dairy herd. High PBI levels were not associated with the feeding of excessive quantities of iodine compounds and were not characterized by high levels of plasma inorganic iodine. Fractionation revealed that, whereas some of the PBI was hormonally inactive, the remainder still indicated an unusually high level of circulating thyroid hormone. However, no symptoms of a pathological hyperthyroid condition could be observed in any of the animals studied. Although grass silage was not the sole cause of the elevation of PBI levels, it was capable of maintaining such levels when, normally, other conditions resulted in their decline.

The occurrence in cattle of either serum or plasma protein-bound iodine (PBI) levels which are considerably above what is generally regarded as the normal has not often been reported. Long *et al.* (7) found PBI values which they characterized as being ten to 20 times normal in cows fed iodinated casein. When the feeding of iodinated casein was discontinued, the PBI levels dropped rapidly but remained above normal for some time. Long, Gilmore, and Hibbs (6) reported PBI values of as high as 70 μg. % as a result of feeding large quantities of potassium iodide to cows. Mixner and Lennon (8) reported increases in plasma PBI levels to as high as 23 μg.% as a result of treating young bulls with thyroid hormones or thyroid-stimulating hormone. In each case, the elevated level of PBI was associated with the intake of large quantities of an iodine compound or with treatment of a thyroid-active substance.

The work reported in this paper was conducted to evaluate the use of plasma PBI as a means of predicting productive potential of dairy cattle. A preliminary phase of the work involved determining whether the variation between animals was significantly greater than the daily and seasonal variation. In the course of this statistical phase, exceptionally high PBI values were frequently obtained. These high values were apparently not related to a high iodine intake nor to any known substances possessing thyroid activity.

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[2] This work was partially supported by funds from Northeastern Regional Project 30, Dairy Cattle Physiology.

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EXPERIMENTAL PROCEDURE

The study of plasma PBI levels was carried out principally with heifer calves and cows of the Reymann Memorial Ayrshire herd of West Virginia University. The nature of the results was confirmed in the Holstein-Friesian and Jersey herds at the same farm. Blood samples were collected from each heifer calf at two-day intervals for either an eight- or 12-day collection period when the animals were 3 and 6 mo. of age. One or two blood samples were also collected from each dam. The blood samples were assayed for plasma PBI. The data obtained from calves at 3 and 6 mo. of age were analyzed for variance according to Snedecor (9).

The milking cows were fed a good-quality mixed hay consisting mostly of orchard grass, Ladino clover, and alfalfa, and either corn or hay-crop silage. They were fed as much roughage as they would consume. They were fed a grain mixture according to production, consisting of 300 lb. wheat bran, 300 lb. corn meal, 500 lb. ground oats, 100 lb. soybean oil meal, 18 lb. salt containing trace minerals, and 12 lb. bone meal.

The calves and heifers were fed a good-quality grass hay consisting mostly of orchard grass and timothy and corn or hay-crop silage. They were fed a grain mixture consisting of 300 lb. each of ground oats, wheat bran, corn meal, and soybean oil meal, together with 18 lb. salt containing some trace minerals and 12 lb. bone meal.

The hay-crop silage of special interest in this study was made in June each year. The forage used came from two fields; one of which consisted of wheat and vetch, and the other of orchard grass, Ladino clover, and alfalfa. The forage from these two fields was mixed in about equal quantities, with wheat and orchardgrass being the predominating grasses. Eight pounds of sodium metabisulfite was added to each ton of silage as a preservative.

The method employed for the determination of plasma PBI was that of Brown, Reingold, and Samson (4), as modified by Lennon and Mixner (5).

Chromatographic separation of the iodine compound bound to the protein of the plasma was accomplished by means of a combination of the methods of Acland (1) and Barna *et al.* (3). The iodine compounds were liberated from the precipitated protein by digestion with barium hydroxide and subsequent extraction with *n*-butanol. The butanol solution was evaporated to a small volume and divided into two equal parts. Carrier quantities of diiodotyrosine and thyroxine were added to one portion and the two parts were spotted in the same manner on different paper strips. The strips were developed with butanol–acetic acid solvent (4:1), dried, and the chromatograph containing the carrier compounds developed with ninhydrin. The corresponding spots on the other chromatograph were cut out and analyzed for iodine content by the same method used for the determination of PBI. Iodine recovered from the spot corresponding to diiodotyrosine was designated as the *D* fraction, and that recovered from the spot corresponding to thyroxine was designated the *T* fraction.

Iodine in whole plasma and in the feedstuffs was determined by the same method used for PBI. It has been pointed out by Acland (1), and confirmed by our experience, that in the use of this procedure iodine not bound to protein

is incompletely recovered. In our work, the recovery of iodine not bound to protein was 75%. In the analyses of iodine not bound to protein, a 75% recovery was assumed and an appropriate correction made.

RESULTS AND DISCUSSION

The mean plasma PBI values of Ayrshire cattle for the specified period of collection are shown in Table 1. Abnormally high PBI values occurred in late winter and spring each year and were also found in the cows during November-December, 1956.

TABLE 1

Mean PBI values of all blood samples collected from Ayrshire females

	PBI (μg. %) for specified period						
Age	Feb.-Mar. 1956	May 1956	Nov.-Dec. 1956	Feb.-Mar. 1957	June 1957	Nov.-Dec. 1957	Feb-Mar. 1958
3 mo.	4.5(20)	.....	7.1(52)	20.4(24)	.....	6.2(24)	19.4(12)
6 mo.	5.5(40)	12.8(16)	.....	25.1(40)	4.6(24)	.....	29.8(24)
Cows	.....	23.1(10)	26.7(12)	21.3(12)	3.2(5)	3.9(6)	25.4(9)

( )—Number of samples.

Between-animal variation was much greater in the calves than in the cows, even though the means are nearly the same. It was noted that 16.6% of the observed PBI values regarded as high in the 3-mo.-old calves were greater than 45.0 μg. %, whereas the corresponding figure for the 6-mo. age groups was only 9.7%. Values of such magnitude were never encountered in cows. The summaries of the analysis of variance for PBI of 3- and 6-mo.-old Ayrshire females are shown in Table 2. It is apparent that the results are quite similar at both ages. Therefore, the relationship of PBI values found in calves and those found in cows eliminates their possible use in predicting the productive potential.

Spot checks of the Holstein-Friesian and Jersey herds indicated quite clearly that the same situation found at a given time in the Ayrshire herd also existed in these herds. It is interesting that at no time could any gross symptoms of hyperthyroidism be detected in any of these animals, despite the fact that values regarded as being from three to ten times above normal were regularly encountered. Age at weaning, onset of puberty, and amount of exposure to sunlight could not be found to have any relationship to plasma PBI levels.

TABLE 2

Summary of analysis of variance of plasma PBI data from 3- and 6-mo.-old Ayrshire females

	Three-month-old		Six-month-old	
Source of variation	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Periods	4	1,015.8**	4	5,246.7**
Individuals within periods	28	474.8**	36	307.4*
Plasma samples	99	12.2	103	16.2
Total	131		143	

* $P < .05$.
** $P < .01$.

During periods when values were elevated, this elevation in PBI values could be shown to have a regular pattern. In Figure 1 are represented the combined daily means of plasma PBI determinations from 3- and 6-mo.-old heifers sampled in February and March, 1957 and 1958. In both years, the values reached a peak in March and then declined. An important difference between the two curves is that in 1957 the same animals were not necessarily sampled on a given day, whereas in 1958 the same group of animals was sampled continuously through the collection period.

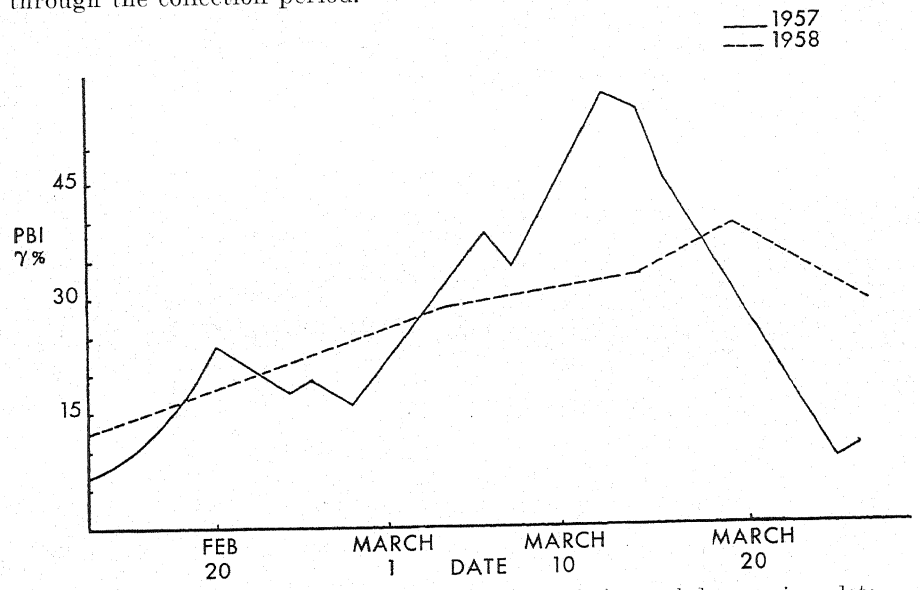


FIG. 1. Mean PBI of all female calves (Ayrshire) sampled on a given date.

The possibility of an error in the PBI analytical method as used in this laboratory was considered. Plasma samples obtained from the West Virginia University Ayrshire herd were submitted to six laboratories for PBI analysis. In four of the laboratories, PBI was analyzed by means of the alkaline ashing procedure and in the other two laboratories the iodine distillation procedure was used. The plasma samples submitted to these laboratories contained both abnormally high and normal PBI values. The results obtained were not significantly different ($P < .05$) from those obtained in this laboratory. These data are presented in Table 3.

The iodine content of the ration ingredients received by these animals is shown in Table 4. None of the ingredients contained appreciable quantities of iodine. From Table 4, it can be calculated that a large dairy cow fed daily 15 lb. of hay, 30 lb. of corn silage (fresh weight), and 15 lb. of concentrate would ingest a daily quantity of only 0.2 mg. of iodine. Long, Gilmore, and Hibbs (6) fed as much as 22 mg. daily of potassium iodide to dairy cows without affecting PBI levels.

According to Long *et al.* (6, 7) and Allcroft *et al.* (2) elevated PBI levels resulting from feeding iodine compound are accompanied by extreme elevation of

TABLE 3

Plasma PBI values obtained in different laboratories using two methods of analysis

	Plasma PBI (μg. %) in sample							
	6[a]	47[b]	52A[b]	52B[b]	63B[b]	65[c]	74[c]	78[c]
A[d]	6.8	12.1	14.4	14.5	....	....	.....	.....
B[d]	7.6	12.3	19.2	21.4	....	....	.....	.....
C[d]	8.1	11.7	20.7	23.3	....	....	.....	.....
D[d]	6.9	10.6	15.8	18.4	....	....	.....	.....
H[e]	....	.....	.....	.....	6.5	7.6	.....	.....
p[e]	....	.....	.....	.....	....	....	17.0	82.7
WVU[d]	6.3	10.8	17.1	16.0	6.3	6.3	15.0	76.0
Mean and S.E.	7.1±0.3	11.5±0.3	17.4±1.2	18.7±1.7				

[a] This was a sample of pooled human plasma purchased from Warner-Chilcott Co., New York.
[b] Plasma obtained from an Ayrshire cow.
[c] Plasma obtained from an Ayrshire calf.
[d] Plasma PBI determined by means of alkaline ashing.
[e] Plasma PBI determined by means of iodine distillation.

TABLE 4

Iodine content of the ration received by the West Virginia University dairy herd

Component	Iodine (μg. per gram)
Concentrate fed to cows	0.008
Concentrate fed to calves	0.009
Hay (fed to milking herd)	0.005
Hay (fed to calves)	0.004
Corn silage (dried)	0.015
Grass silage (dried)	0.009

the level of iodine in plasma not bound to protein (plasma inorganic iodine). They reported that PBI levels of 30 to 70 μg. % are accompanied by inorganic iodine levels of from 400 to 1,100 μg. %. According to these workers, PBI would represent about 10% of the total blood iodine. It can be seen from Table 5 that with samples chosen at random from those collected in this study, in every case the PBI represented more than 90% of the total blood iodine.

The division of PBI into the *D* and *T* fractions is presumably a division into hormonally inactive and active fractions, respectively. Long, Gilmore, and Hibbs (6) have suggested that in the case of high PBI values in which no symp-

TABLE 5

A comparison of plasma PBI and total plasma iodine of Ayrshire females

PBI	Total iodine	PBI % of total iodine
(μg. %)	*(μg. %)*	
7.0	7.8	89.7
5.4	6.1	88.5
6.6	7.4	89.2
4.8	4.9	98.0
16.3	18.8	86.7
29.0	32.3	89.8
38.7	40.9	94.6
52.8	57.0	92.6
Mean	.....	91.1

toms of hyperthyroidism were present a large part of this PBI was represented by hormone-like iodination products of protein, such as the hormonally inactive products in iodinated casein. It would be expected that such products would be in the *D* fraction and active compounds would be in the *T* fraction. In Table 6 it may be seen that, in contrast to normal PBI levels, high PBI levels are characterized by a considerable percentage of iodine in the *D* fraction. However, the level in the *T* fraction is much higher than that regarded as normal and should reflect a pathological thyroid state which was not in evidence in this study.

TABLE 6

Chromatographic fractionation of PBI from cattle with high and normal levels. Values expressed in micrograms per 100 ml. of plasma

Sample type	*D* fraction	*T* fraction	PBI	Per cent recovery
High	14.5	21.1	44.0	81
High	16.0	28.8	60.1	75
Normal	0	3.8	4.5	84
Normal	0	3.8	4.1	93

The appearance of high plasma PBI values corresponded quite closely to the time at which grass silage feeding was begun. In the fall of 1956, the cows were fed all grass silage in contrast to the fall of 1957, when both corn and grass silage were fed. In the fall of 1956, one 9-mo.-old heifer which had received grass silage had a mean plasma PBI for four collections of 48.3, compared to a mean of 8.2 for four other animals of the same age which had not received silage. In the spring, the rise of the plasma PBI of calves corresponded approximately to the beginning of grass silage feeding. It would appear that while grass silage is not the sole cause of the elevation in PBI, it is a contributing factor of some importance.

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TECHNICAL NOTE

EVALUATION OF SHORT-INTERVAL MILKING AS A PHYSIOLOGICAL TECHNIQUE

While attempting to evaluate short-interval milking as an experimental approach to problems in milk secretion in dairy cattle, Lakshmanan *et al.* (1) observed that certain factors appeared to influence the amount of milk and butterfat produced during two-hourly milking for a short period. Because of the possible importance of this technique, we should like to make the following comments on those aspects of the paper which we feel may lead to ambiguity in interpretation.

1. Production in any 24-hr. period can not be regarded as a valid response criterion; hence, the analyses of variance presented are based on "observations within plots" and are invalid. The three "experiments" were, in fact, three uniformity trials; the three observations on each cow provide only a single measure of the response to short-interval milking. Thus, two reasonable measures of response per cow would be,

$$R = \text{Period II} - \left(\frac{\text{Period I} + \text{Period III}}{2}\right)$$

or alternately,

$$R = \frac{2\,\text{Period II}}{\text{Period I} + \text{Period III}}$$

That the three observations provide only one piece of information is obvious when it is considered that if, for any cow, the Period II observations were lost, the remaining observations on that cow would be meaningless.

2. The general procedure in controlled experimentation is to apply treatments to experimental units and to measure the resulting yield. In Lakshmanan *et al.* (1), the experimental units were cows, the treatments: (a) high producers early in lactation; (b) low producers early in lactation; (c) high producers late in lactation; and the yield was response to two-hourly milking. With 13 cows, their data had a total of 12 degrees of freedom, for which the correct analysis of variance would be:

Source of variation	*DF*
Between treatments	2
Error (cows within treatments)	10
Total	12

This analysis, of course, would require all cows to be milked under the same conditions. In fact, however, treatment (b) was carried out at a different time than the other treatments; hence, its effect was confounded with the different shed conditions prevailing at the time. Nevertheless, accepting this restriction, it is clear that the authors could have carried out a valid statistical test of the hypothesis that the potential level of production per cow influenced the response to short-interval milking.

3. The simplest hypothesis on the response to frequent milking would probably be that at a particular stage in lactation each cow has a potential production, P, but that when milked, for example twice-daily, the cow produces an amount, T, less than or equal to P. Yield of potentially low-producing cows at all stages of lactation is unlikely to be influenced by frequent milking, hence,

$$T = P \qquad (1)$$

At higher levels of potential production, however, twice-daily milking might be expected to limit actual production to a fraction of potential production, that is,

when $T < P$,

$$T = a + bP \qquad (2)$$

where $0 < b < 1$.

Taking two-hourly milking for 24 hr. as a measure of potential production, P, and twice-daily production as a measure of T, the following estimate of Equation (2) was obtained from the present data by regression:

$$T = 7.99 + 0.7755\,P. \qquad (2')$$

This relation, which has a coefficient of determination, r^2, of .96 is shown graphically in Figure 1, where the maximum value of P equal to T is estimated to be 35.6 lb. of milk per 24 hr.

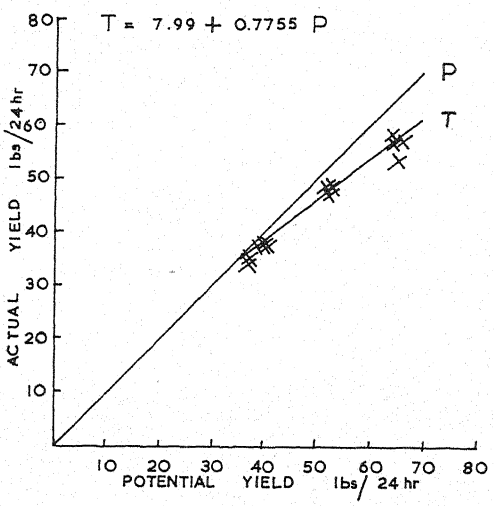


FIG. 1. The relationship between actual and potential yield in twice-daily milking.

In computing (2′), the data for the cow in its first month of lactation were omitted, because the relationship between actual and potential yield in early lactation is complicated by the unknown factors responsible for the positive slope of the lactation curve in early lactation.

It should be noted that the regression technique used to compute (2′) is open to criticism because of the identity,

$$P = T + \text{difference}.$$

Nevertheless, (2′) is probably the most illuminating use that can be made of the available data.

4. No justification is given for selecting a period in which the cows are taken to be adjusting to the new milking regimes. We may observe that since the mean yield for Period III was greater than for Period I, and since the cows were nearer to the end of their lactation in Period III, it is possible that an overcompensation by each cow took place. If shown to be true, a finding such as this would have great physiological significance. To test accurately for compensation, it is necessary to take into account the regression of time on yield (i.e., on the slope of the lactation curve). This might be accomplished through using a slope calculated from a large population of cows or, alternately, a more simple method might be a comparison of the slopes in the two periods.

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OUR INDUSTRY TODAY

DRY VS. LYE-SOLUTION STORAGE OF MILKING MACHINE RUBBER INFLATIONS[1]

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Milking machine rubber inflations deteriorate at an accelerated rate as a result of the absorption of fats during milking. This fat absorption and extraction by and from various natural and synthetic rubbers have been studied by several workers (1-5) who, in general, showed the damaging effects of fat on rubber and the benefits of lye storage.

Milking machine inflations undergo changes in size, shape, and composition as a result of use and storage during use interims. The rapidity with which these changes take place determines the useful life of inflations. If this period of usefulness could be prolonged by improved storage conditions, the savings effected thereby to the milk producers would be of incalculable value. To this end, a long-range, farm-use storage study was made with different brands of natural and Perbunan rubber inflations. The effects of dry vs. lye-solution storage on the deterioration and life of milking machine rubber inflations were compared.

HISTORICAL

Investigations dealing with the deterioration of rubber by absorption of fats and removal of fats with caustic soda, thereby extending its life, have been conducted by workers in New Zealand and England as well as in the United States (1-10). Fat absorption has been shown to take place largely during the time of milking, with approximately half of the fat absorbed from the milk and half from the skin and hair of the cows.

Deterioration of rubber occurs in two stages: namely, the primary, causing softening and swelling and the secondary, resulting in oxidation and hardening of the rubber. The primary types of deterioration are largely reversible, but the secondary ones are irreversible.

Laboratory studies, as well as field studies, show that fats are effectively and conveniently removed by storing inflations in 5–10% strength solutions of cold caustic soda for periods of 1-2 wk. With this procedure, Gardner and Berridge (4) noted a 50% increase in the useful life of inflations.

SCOPE OF THE INVESTIGATION

This study on dry vs. lye-solution storage of milking machine rubber inflations was made under actual field conditions. Both natural and synthetic rubber inflations were subjected to 6 mo. use for each of the two methods of storage. The year-long investigation, embracing all-season conditions, involved 36 milk producers, who used the rubber inflations according to specific directions. The study encompassed 360 natural and 232 synthetic rubber inflations, which were inspected intermittently throughout the year and checked carefully at the end of the use period.

EXPERIMENTAL PROCEDURE

Milk producers in Central Michigan were selected for the study according to routes. Producers were asked to use sets of milking-machine inflations for a week, then replace the inflations and store them either dry or in a 5% lye solution, as the experiment dictated. During use, inflations were stored dry between milkings. Producers washed their inflations without specific instruction. All inflations placed on all the milking machines were new and branded with their respective weights. Each producer was given two sets of inflations so they could be alternated at weekly intervals. Extra inflations, identified by special marking, were furnished for replacing damaged ones.

The inflations used were of standard, natural rubber and of Perbunan rubber quality as furnished by various manufacturers.

Equipment for lye storage consisted of containers, racks, and lye was provided. Earthenware crocks were used at the start of the study but were replaced later with 5-gal. steel pails.

Producers were requested to place tags on inflations which required removal from service, with notation of date of discontinuance. At the end of 1 yr., or 6 mo. use for each set of inflations, all the inflations were picked up for reweighing and laboratory examination.

RESULTS

Effects of dry storage. Data presented in Table 1 show the effects of storing inflations dry for seven-day periods after each week of use.

All the natural rubber inflations, regardless of brand, increased in weight. The increase in weight per brand of inflation ranged from 7.4–15.8%. The average increase in weight was 10.8 g. per inflation, or 10.4% of the original inflation weight.

All Perbunan inflations lost weight equal to 1.73% of the original weight. The percentage

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TABLE 1

Weight changes of natural and synthetic rubber inflations during 12 mo. of use when stored dry during alternate weeks

Brands of rubber used	Cows milked (No.)	Average initial weight of inflations (g.)	Change in weight of inflations (g.)	Change in weight of inflations (%)
		Natural rubber		
B1	3,600	87.3	+7.2	8.2
B2	3,832	87.1	+10.4	11.9
F1	3,280	123.7	+9.2	7.4
F2	4,015	123.4	+14.4	11.7
F3	2,525	124.0	+10.4	8.4
A	2,525	92.1	+14.6	15.8
D	3,030	90.8	+7.4	8.1
		Average	10.8	10.4
		Perbunan rubber		
H1	2,555	87.4	−1.5	−1.7
H2	1,642	87.5	−0.8	−0.9
H3	3,650	87.3	−1.3	−1.5
H4	2,007	87.4	−1.1	−1.3
H5	3,650	86.6	−1.2	−1.4
H6	2,900	86.5	−1.5	−1.7
H7	3,100	88.0	−3.2	−3.6
		Average	−1.05	−1.73

loss of the inflations ranged from 0.9–3.6% per inflation.

Of 136 natural rubber inflations used 97, or 71.3%, were discarded during the period of study; whereas, only 16 of 120 Perbunan inflations, 13.3%, were discarded during a similar period (Table 2).

Effects of lye-solution storage. Data presented in Table 3 show the effects of 5% lye solution on weight changes of milker inflations used during the 12-mo. study.

The natural-rubber inflations gained on an average 3.94 g. per inflation, or 3.87%. Except for Brand D, which lost 2.1 g. per inflation, the gains ranged from 2.8–7.2 g.

The Perbunan inflations lost weight with lye storage; the average loss was 7.1 g. each, or 8.1%. The losses ranged from 6.7 to 9.5%.

Sixty-five of the 159 natural rubber inflations used and stored in lye, or 28.1%, were discarded as a result of damages sustained by them (Table 4). Damage consisted of roughness inside inflation barrels in 48 inflations, and from stem tears and punctures in 17 inflations.

Reasons for discarding inflations. Data presented in Table 5 show the nature and extent of damage to natural and Perbunan rubber inflations under dry and lye storage.

Of 136 natural-rubber inflations subjected to alternate-week dry storage 97, or 71.3%, were discarded. Forty-three inflations, 31.6% of the total number used, were discarded because of stem damage, and 54, or 40%, because of damage to inflation barrels. Thus, of 97 natural-rubber inflations discarded, 44% had stem damage and 56 had barrel damage.

Of 224 natural-rubber inflations subjected to lye storage 65, or 29%, were discarded. Only 8.5% of the total number used were discarded because of stem damage, and 20.5% because of barrel damage. Thirty per cent of those discarded were stem-damaged and 70% were barrel-damaged.

TABLE 2

Milking-machine rubber inflations discarded during 12 mo. of use when stored dry during alternate weeks

Brands of rubber used	Cows milked (No.)	Inflations			
		At start of study (No.)	At end of study (No.)	Discarded during study (No.)	Discarded during study (%)
			Natural rubber		
B1	3,600	24	0	24	100.0
B2	3,832	32	23	9	28.0
F1	3,280	16	0	16	100.0
F2	4,015	24	0	24	100.0
F3	2,525	8	8	0	0.0
A	2,525	8	8	0	0.0
D	3,030	24	0	24	100.0
	Total	136	39	97	71.3
			Perbunan rubber		
H1	2,555	16	15	1	6.3
H2	1,642	16	16	0	0.0
H3	3,650	16	12	4	25.0
H4	2,007	16	13	3	18.8
H5	3,650	24	20	4	16.7
H6	2,900	16	15	1	6.3
H7	3,100	16	13	3	18.8
	Total	120	104	16	13.3

TABLE 3

Weight changes of natural and synthetic rubber inflations during 12 mo. of use when stored alternate weeks in a 5% lye solution

Brands of rubber used	Cows milked (No.)	Average initial weight of inflations (g.)	Change in weight of inflations (g.)	(%)
		Natural rubber		
A1	912	90.3	+5.1	5.6
A2	1,825	92.0	+4.4	4.8
A3	3,285	92.0	+7.2	7.8
B1	4,197	87.1	+3.1	3.6
B2	2,920	86.8	+2.8	3.2
C1	2,525	98.6	+3.5	3.5
C2	1,825	98.9	+4.9	4.9
D	1,825	91.1	−2.1	−2.3
E1	1,896	99.8	+4.8	4.8
E2	1,825	106.5	+6.0	5.6
F1	3,650	122.8	+3.1	2.5
F2	2,920	123.5	+3.5	2.8
F3	2,190	123.2	+5.4	4.4
G	1,581	113.7	+3.5	3.1
		Average	3.94	3.87
		Perbunan rubber		
H1	912	97.2	−6.5	6.7
H2	1,460	88.4	−8.4	9.5
H3	1,825	87.9	−7.1	8.1
H4	2,723	87.1	−6.8	−7.8
H5	1,277	80.0	−6.5	−8.1
H6	2,190	86.9	−7.4	−8.5
H7	3,650	81.9	−7.0	−8.5
H8	3,467	86.8	−6.9	−7.9
		Average	−7.1	−8.1

Of 120 dry-stored Perbunan inflations 16, or 13.3%, were discarded because of stem damage. Of 112 lye-stored Perbunan inflations 12, or 10.07%, were discarded for the same reasons. No Perbunan inflations were discarded because of barrel damage.

Appearance of inflations at close of study. Natural rubber inflations differed widely in appearance after storage, depending upon the type of storage. The dry-stored inflations became longer, of greater diameter, and appeared distended and swollen. Furthermore, many inflations were misshapen, to the extent that when placed on end they did not stand upright (Figure 1).

On the other hand, those inflations stored in the 5% lye solution retained their original shape and size. All natural-rubber inflations, as a whole, were equally rough as determined by feel and appearance.

Both Perbunan and natural rubber inflations, after several weeks of dry storage, were coated with a grayish film of fat which had been expressed by the rubber. The heaviest coating of film appeared in the flexing portion of the inflation barrel. No such coating appeared on those inflations stored in lye solution. Lye-stored inflations retained a clean, fresh, new appearance throughout the study.

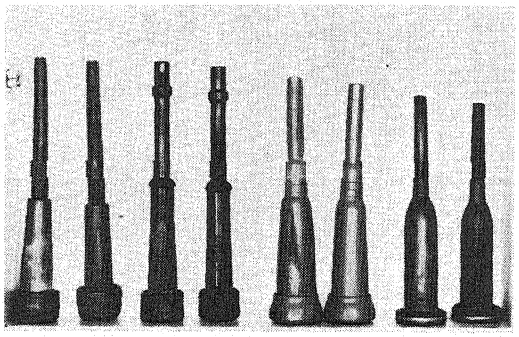


FIG. 1. Showing differences in appearance and size of inflations, following dry storage (left one of each pair) and lye storage.

Perbunan inflations retained their original shape, regardless of the type of storage. Indications of swelling or deterioration of the Perbunan were not noted. However, some roughness inside the barrels was observed. The lye-stored Perbunan inflations were damaged slightly more than were those stored dry. Also, the lye-stored inflations shrank in over-all size to a small extent. Presumably, the shrinkage was equal to the difference between the losses in weight when the inflations were stored under either of the two conditions. Therefore, the Perbunan inflations under lye storage were presumed to be approximately 6.5% smaller than those dry stored.

A further outstanding difference resulting from the type of storage was the odor of the inflations. Regardless of the type of inflation, dry storage yielded an unclean odor. This was in contrast to the very clean, fresh odor found in those inflations stored in lye solution.

Containers for lye storage. New, earthenware 5-gal. crocks were supplied producers using the lye-storage treatment of milking-machine rubber inflations. After about 2 mo. of use, lye solution seepage occurred, due to the lye destroying the glazed finish on the crocks. The crocks were then replaced with 5-gal., 24-gauge steel pails. The pails selected were fitted with gasketed covers which were closed by a level-locking ring. For convenience in handling, the pails were provided with bails.

The steel pails proved satisfactory for lye storage. They did not leak and they were not subject to lye corrosion. Some rust spots appeared on the underside of the cover, resulting from condensation of moisture. The steel pails were easy to handle and, being covered, gave protection against entry of soiling matter. The element of danger in the use of strong lye in an open container was avoided by using a locking cover. The kind of pail used is shown in Figure 2, along with an inner polyethylene pail to hold the inflations. The polyethylene pail was punched with holes, making it serve as a colander, for rapid entry and discharge of the lye solution.

TABLE 4

Milking machine rubber inflations discarded during 12 mo. of use when stored alternate weeks in a 5% lye solution

Brands of rubber used	Cows milked (No.)	Inflations			
		At start of study (No.)	At end of study (No.)	Discarded during study	
				(No.)	(%)
Natural rubber					
A1	912	8	6	2	25.0
A2	1,825	16	15	1	6.3
A3	3,285	16	16	0	0.0
B1	4,197	16	14	2	12.5
B2	2,920	16	16	0	0.0
C1	2,525	16	8	8	50.0
C2	1,825	8	7	1	12.5
D	1,825	16	16	0	0.0
E1	1,896	24	0	24	100.0
E2	1,825	16	14	2	12.5
F1	3,650	16	12	4	25.0
F2	2,920	16	16	0	0.0
F3	2,190	24	19	5	20.8
G	1,581	16	0	16	100.0
	Total	224	159	65	29.0
Perbunan rubber					
H1	912	8	8	0	0.0
H2	1,460	16	13	3	18.8
H3	1,825	16	16	0	0.0
H4	2,723	16	14	2	12.5
H5	1,277	8	7	1	12.5
H6	2,190	16	12	4	25.0
H7	3,650	16	14	2	12.5
H8	3,467	16	16	0	0.0
	Total	112	100	12	10.7

TABLE 5

Nature of damage to natural and synthetic rubber inflations when subjected to dry vs. lye storage

Type of rubber	Kind of storage	Inflations			Inflations discarded because of					
		Used (No.)	Discarded		Stem damage			Barrel damage		
			(No.)	(%)	(No.)	(%)	(%)[a]	(No.)	(%)	(%)[a]
Natural	Dry	136	97	71.3	43	31.6	44.0	54	40.0	56.0
Natural	Lye	224	65	29.0	19	8.5	30.0	46	20.5	70.0
Perbunan	Dry	120	16	13.3	16	13.3	100.0	....	.....	.....
Perbunan	Lye	112	12	10.7	12	10.7	100.0	....	.....	.....

[a] Percentage of total discards.

DISCUSSION

The seven-day, alternate-week storage in 5% lye solution was effective in prolonging the useful life of inflations, the retention of their original shape and size, as well as in keeping them fresh in odor and clean in appearance. While natural rubber was benefited by lye storage more than was Perbunan, both kinds of inflations were made clean and fresh of odor to an extent that was never attained by the best washing practices.

An increase in weight of inflations was recognized as being caused by fat having been absorbed from the milk and from the skin of the cow's udder, the absorption taking place mainly during milking. That portion of the inflation subjected to flexing with the pulsation of the milker absorbed the greatest amount of fat. Apparently, once flexing was started in an area of an inflation, the pattern of flexation became established and continued there despite removal of inflations for cleaning after each milking. Absorption of fat occurred also in nonflexing portions of the inflations, as observed by films of fat which appeared on idle inflations after several weeks of storage.

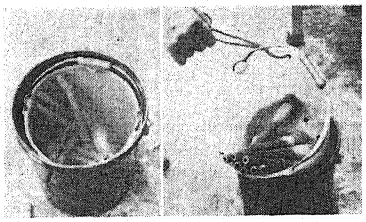


FIG. 2. Showing use of lye storage containers.

Natural rubber inflations increased in weight with absorption of fat. Perbunan, on the other hand, showed loss in weight. A similar characteristic of Perbunan was shown in a previous investigation (5), wherein loss of weight was experienced when Perbunan was soaked in butter oil. Perbunan inflations, nevertheless, absorbed some fat as indicated by an appearance of fat films on inflations held in the laboratory several weeks at the completion of the study.

SUMMARY AND CONCLUSION

Natural and Perbunan rubber milking machine inflations were stored dry and in a 5%, cold-lye solution during alternate weeks under field conditions for a year to compare their lasting qualities, weight changes, and appearances.

With dry storage, the natural rubber inflations gained an average of 10.4% in weight. Similarly stored Perbunan inflations lost weight, averaging 1.73%.

When stored in the lye solution, the natural rubber inflations increased in weight, averaging 3.87%. Under similar conditions of storage, the Perbunan inflations lost weight, averaging 8.1%.

A higher percentage of natural rubber inflations, 71.3% as compared with 29%, was discarded when the inflations were stored dry rather than in lye solution. Perbunan inflations were equally durable under either condition of storage.

Lye-solution storage favored retention of shape and quality of the natural rubber inflations, whereas those dry-stored became enlarged and misshapen with use.

Lye-solution storage yielded cleaner appearing and sweeter-smelling inflations than dry storage, regardless of the type of inflation.

ACKNOWLEDGMENT

The author gratefully acknowledges financial support and assistance with the inflation materials used in this study by the United States Rubber Company, Passaic, New Jersey; also, to Ira Wilson & Sons Dairy Company, Detroit, for cooperation with producer sampling.

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RECENT DEVELOPMENTS IN AUTOMATIC CLEANING OF STORAGE TANKS[1]

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Automatic cleaning means mechanical cleaning. When successful mechanical cleaning is being done it is possible, by instrumentation and control, to automatize the cleaning system to any desired degree. Different ideas seem to exist as to what sanitation means as it applies to dairy equipment. In this discussion we mean a tank that is clean as demanded by all sanitary standards. Several devices have been used to clean storage tanks by mechanical methods.

[1] Presented at Seventh Annual National Dairy Engineering Conference, Michigan State University, East Lansing, February 26-27, 1959.

We shall examine these devices and see how each one is supposed to operate.

EQUIPMENT

Spray balls. More spray balls have been installed in storage tanks than any other device. Some have been installed as a permanent part of tank equipment. Others have been designed to be placed in the tank when the cleaning operation is started. Both types of spray balls are expected to do the same cleaning job, so they will be grouped together. These spray balls have been placed on piping extending down from the top of the tanks, on horizontal piping welded to the tank liner, and on piping that rests on supports standing on the bottom of the tank. They have generally been self-draining. In fact, they need to be self-draining and self-cleaning if they are to be successful. Some spray balls are made in two sections which are separable and can be taken apart for cleaning. Other spray balls have a plastic ball installed inside, made to bounce around by the solution flow and keep the inside of the ball clean.

Recently, variations of the spray ball have been designed and installed in storage tanks. Generally, they are not spherical in shape, but are supposed to do the same job as the regular spray balls.

The spray balls and variations of them have given varying results. A pump to supply pressure to the cleaning solutions has been attached to the spray ball piping system. These spray balls have been used in all sizes and shapes of tanks. Generally speaking, a single spray ball has been installed for each 8 ft. of tank length. This has been a general rule, but is not true in all cases. They have been placed at different distances from the bottom and from the top of the tanks.

Hot sprays. The second device that has been used is the hot spray unit. This consists of a tank, hose, nozzle, and pump. A solution is prepared in the tank, and it is heated and sprayed on the tank surface. The hot solution is allowed to remain on the surface for a short time. A respray with the hot solution follows and then rinsing with fresh water as the last treatment. A man must enter the tank to apply the cleaning solution properly.

Injector sprays. The third cleaning device is the injector type of hot spray unit. This consists of a detergent reservoir tank, a Venturi element, and a mixing valve with hose and nozzle. The tank is filled with detergent and connected to a water supply. The water flows through the Venturi and draws detergent from the supply tank and mixes the solution. The nozzle is then controlled by the man who sprays the surface to be cleaned.

Rotary sprayers. The fourth device is the rotary type sprayer. It has a rotating head, driven by a motor, fitted with a number of spray nozzles. These nozzles are supposed to overlap and completely cover the inside surfaces of the tank. This sprayer requires a pump to circulate the cleaning solution through the spray nozzles.

Rotating nozzles. The fifth device consists of a rotating nozzle-equipped head. It is rotated by the jet action of one or more of the spray nozzles. This device has the spray patterns designed to overlap on the tank surface. The rotating mechanism has been made with sleeve type bearings and, more recently, with stainless steel ball bearings. This unit requires a fixed pressure system, to insure positive and constant speed of rotation. Usually, it has been installed in the top of storage tanks. Not many of these cleaning devices have been installed in tanks.

Rotating shafts. The sixth cleaning device consists of a rotating hollow shaft that extends down through the top of the storage tank. A fan-shaped, slotted jet is attached to the hollow shaft. Cleaning solutions, under pressure, flow through the hollow shaft and out through the slotted jet. Because the slotted jet throws a fan-shaped pattern of cleaning solution, an uninterrupted stream of solution is placed on the top surface of the tank liner. As the jet assembly rotates, complete coverage of the tank interior is accomplished with each revolution of the jet. Normally, this mechanism rotates five times per minute. This means that five times per minute the entire interior of the tank is covered with cleaning solution.

This rotating slotted jet has also been installed as an integral part of the agitator shaft. It is designed to allow normal use of the agitator without any adjustment. When the cleaning operation is started, solution is pumped through an inlet on top of the tank. The cleaning solution flows through the hollow shaft and out through the slotted jet. This fan-shaped pattern of solution covers the upper surfaces to be cleaned and flows down the sides and ends. An opening in the device allows solution to flow down the agitator shaft and thoroughly clean the shaft and upper blade or blades. The bottom agitator blade, or blades, and the bottom bearing, are cleaned by rotating in the pool of cleaning solution that collects on the bottom of the tank. This solution is drawn out through the outlet and discharged to the drain or used again.

These devices have been used for cleaning storage tanks, and different ideas exist as to their cleaning efficiency.

PROCEDURES

Probably the best way to clean a storage tank by mechanical means would be to proceed as follows:

1. Fill the tank with water, add the proper chemical to make a good cleaning solution, heat the solution to the proper temperature, and shake the tank until the soil is removed from the cleanable surfaces. The only problem is to

find a man strong enough to pick up a storage tank and shake it. By accepting this method as the perfect one, we can then consider these mechanical cleaning devices and see how they approach perfection. Certainly, it is safe to assume that good chemicals are available to do the cleaning job. Water and methods of heating are at hand. Pumps, tanks, and controls are all presently available. The different devices can all easily be installed in tanks. Now, all that remains is for the mechanical devices to completely cover the surfaces to be cleaned with cleaning solution.

The Spray Ball Method can do this, provided it is properly installed and if the proper pressure and volume of cleaning solution is supplied to the inlet. Also provided, and this is very important, that the storage tank has been designed to be cleaned by mechanical means. A spray ball, 4 in. in diameter, can have any number of holes drilled into its surface, through which the cleaning solution is discharged. To work at all, these balls must be some distance from the surface to be covered. Otherwise, all the solution would strike the surface in a small area. As the distance increases between the spray ball and the surface to be cleaned, the streams of solution get farther apart. This means that the solution has to bounce or slide across the area between these impact points. It is quite difficult to control this bounce or slide and, again, proper installation should be stressed. If the tank has a vertical agitator shaft, it is difficult to spray the back side of the shaft. It is hard to make water bounce, slide, or go around corners, into blind spots, bearings, or around any obstructions in the tank. The results from spray ball installations indicate that extreme care must be taken to insure that successful cleaning will be realized.

The hot spray system of cleaning tanks can be efficient, but it is still a manual operation. It requires the same attention to chemical solution, temperature, and time to insure success. This operation also requires that a man go inside the tank, which limits its usefulness as a mechanical method of cleaning storage tanks.

The injector system of hot spraying is essentially the same as the pump type system mentioned previously.

It should be mentioned that some air-agitated tanks have been fitted with pumps, to force cleaning solution through the air tubes, and that this was supposed to clean these tanks, but there is no information on the efficiency of this method. If complete coverage of the tank interior can be accomplished, this method could be successful.

The rotating head fitted with spray nozzles can be installed in tanks. This device will clean tanks, if it is supplied with the proper chemical solution, under the ideal pressure. This device has limitations. It can not spray around corners or behind shafts. It must be carefully engineered and installed. The nozzles must be adjusted to overlap and completely cover the soiled surface. The problem of being self-draining and sanitary in design must be met. Ordinary pipe threads on the nozzles must be removed and a sanitary connection made. This rotating nozzle-equipped head must be carefully installed in a tank to be efficient.

The self-rotating cleaning head has the same problems in cleaning tanks as do the other devices, and involves the design of the rotating mechanism. The bearings must be sanitary, self-cleaning, and self-draining. Cleaning solutions and the temperature of them affect the operation of this device. Because it is jet-operated, pressure affects its speed. This is another device that can clean tanks if all the engineering problems are solved and if it is properly installed in a tank that can be cleaned by mechanical means.

The slotted jet mechanism that is rotated by a separate motor will clean tanks with horizontal agitators or air agitation, if properly installed. The solution comes from the jet near the top of the tank. The jet puts solution on the top surface to be cleaned and into the sight-glass and vent openings. The solution flows down the sides of the tank to clean these surfaces. The device requires that a 4-in. sanitary ferrule be welded into the top of the tank. The mechanism attaches to this ferrule with a 4-in. sanitary nut. It is self-supporting and the motor is part of the unit. This makes a sanitary, easily inspected device, as the slotted jet comes apart for visual inspection.

The slotted jet device has given excellent cleaning results when installed on a vertical agitator shaft. The tank is cleaned because the slotted jet completely covers the inside of the tank, provided it has been designed to be cleaned by mechanical means.

Care must be exercised that this device is not rotated too fast. At 36 r.p.m. the device will clean a tank 16 ft. long. Longer tanks require slower-speed agitator motors. Two-speed motors have solved this problem.

Some tanks are not made to be cleaned mechanically. It is better to admit this fact and save trouble and misunderstanding when the proper results are not obtained.

From this evaluation of the available mechanical cleaning methods, it is apparent that a good practical method of cleaning storage tanks can be developed to a point where success is assured.

Along with the description of recent cleaning-device developments should go a description of cleaning methods as related to chemicals, time, temperature, rinsing, sanitizing, and volume of solutions required. Much work has been done along these lines and some practical answers to the questions have been developed.

Prerinsing. We have found that a good prerinse with water at 90 to 100° F. is the best way to remove the loose soil in storage tanks. Applying three-tenths of a gallon to each square foot

of tank surface will result in good prerinsing. This figure is correct with a slotted, fan-shaped jet. A good practical way of deciding on the amount of prerinse water to use is to observe the water as it leaves the tank outlet. When it is clear, the tank is adequately rinsed.

Chemicals. We recommend that the advice of chemical manufacturers be followed. These people know their products and what they will do. They have the materials to properly clean these tanks by mechanical means, and they should be consulted in their use. The solution must be strong enough to remove the soil in the tank, but excessive chemicals result only in waste. We feel that the solution temperature should be kept as low as possible. However, the chemical in question may require a minimum temperature, so it is best to follow recommendations. Remember that heat causes the stainless steel to expand, cold causes it to contract. These forces should be held in check. Results have proven that cleaning can be accomplished by a maximum solution temperature of 135° F. The slotted jet delivering 50 g.p.m. to the interior surfaces of the tank will clean a 2,000-gal. storage tank in 10 min.

A cooling fresh-water rinse should be applied after the cleaning solution treatment. This removes all traces of the solution and cools the tank surfaces. Good rinsing is essential to good cleaning.

DISCUSSION

Mechanical cleaning to be successful depends on these factors: complete coverage of the surfaces to be cleaned with a chemical solution of the proper concentration, heated to and maintained at the proper temperature. The solution must be kept in contact with the tank surfaces for the prescribed length of time. Coverage, time, temperature, and solution concentration are the things necessary to insure clean tanks.

An important side-effect to consider when cleaning storage tanks is the cooling system on the individual tank. Hot solutions contacting the refrigerated zone can cause much grief, if the proper precautions are not followed. It is essential that a refrigeration engineer be consulted before proceeding with mechanical cleaning.

From developments up to this time, it is apparent that we can clean storage tanks by mechanical means. The system can be automatic to any desired degree. Installing an automatic circulation cleaning system insures that time, temperature, and solution concentration are maintained. Installing flow-diversion valves in the lines to and from the tanks to be cleaned, and applying controls and indicators to them, will make a system push-button in operation.

In other words, pushing button No. 1 would clean Tank No. 1 automatically. All precautions would be observed by the control system. If the tank was not empty, a control would not allow the cleaning operation to begin. Anything desired can be built into the automatic system.

These recent developments have indicated that a practical method of cleaning tanks mechanically has been developed to the point where success is assured.

These developments have proven that mechanical cleaning is being accepted by the industry. It is important to build storage tanks with mechanical cleaning as the main objective. This requires some thorough planning by the designers and means that we must consider new ideas about the construction of storage tanks. It is very difficult to apply a good mechanical cleaning device to a tank built years ago for hand-cleaning.

The outlet of the storage tank can generally be cleaned mechanically. The manhole, door, and gasket on present tanks must be washed by hand. This is true when mechanical cleaners are used. The sight-glass opening on most tanks has to be removed and hand-washed; the same is true of the inlet and air vent openings. The test valve, the measuring device on the tank, and the agitator bearing must be cleaned by hand. Therefore, it is apparent that consideration must be given to the redesigning of tanks. It is possible to build a storage tank now that can be cleaned mechanically. This could be done by moving the manhole to the top of the tank. The manhole would be used only for inspection and could be much lighter and would be out of the product zone. The air vent and the product inlet could be in this door or, in many cases, the product could enter the tank through the outlet valve. This method would remove the product inlet and its cleaning problem. A practical method of measuring the volume of liquid in the tank is available and it is known as the bubble system; it requires only a small sanitary C.I.P. fitting on the bottom of the tank. The indicating thermometer fitting is no problem. The test cock may have to be cleaned by hand, if it is necessary to have one on the tank. If a sight-glass is necessary, it can be of the new flush-mounted cleaning-in-place type that is easily cleaned. The vertical agitator has the cleaning device mounted as an integral part and is self-cleaning and its support bearing is also self-cleaning.

When tanks are built along these lines, mechanical cleaning will be easy and positive results will be obtained. These cleaning devices are developed and are practical. Storage tanks must be built to take advantage of these labor-saving methods.

OUR ASSOCIATION

CALL FOR PAPERS FOR THE 1960 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

N. L. JACOBSON, Chairman, Program Committee
Department of Animal Husbandry, Iowa State College, Ames

The 55th Annual Meeting of the American Dairy Science Association will be held June 19-22 at Utah State University, Logan. Members who wish to present papers must submit titles and abstracts not later than March 1. This is a change from last year. The new deadline must be met to permit publication of titles in the May issue and abstracts in the June issue of the JOURNAL.

Abstracts must contain no more than 200 words, arrived at by counting each typed line as 12 words. Abstracts longer than 200 words will be returned to the author(s) by the Committee. An original (on bond paper) and three copies of each abstract should be typed double-spaced. The original and one copy should be mailed to the Chairman, another copy to the Vice-Chairman, and the fourth to the Secretary of the Section before which the paper is to be presented.

In the preparation of abstracts, the style and abbreviations of the JOURNAL OF DAIRY SCIENCE must be used. Please refer to abstracts in the May, 1959, issue of the JOURNAL for guidance. The title of the abstract should indicate clearly the nature of the research. The abstract should include, insofar as possible, the design and major results of the study. Only research which has been completed should be reported. Brief, essential statistics will make the data more meaningful.

All members of the Association are encouraged to present papers. Participation by members in the industry and by senior members of the Association is particularly welcome. The competitive presentation of papers by graduate students, initiated in the Production Section in 1959, will be extended both to the Manufacturing and to the Production Sections in 1960.

Abstracts of research accepted for publication by a scientific journal before the annual meeting are not acceptable. The Program Committee favors the general policy that an individual present only one paper and that his name appear as author on no more than two. The Program Committee, with the Association members, desires to stimulate vigorous, sound research and realizes that the above restrictions may penalize some members engaged in full-time research. Therefore, the Committee has liberalized this policy so that a member can exceed these limits, but only if he or his department rates the abstracts in order of preference for oral presentation. If the total number of papers submitted by the membership is too great to include in the program, the Committtee will assign some papers to be read by title only.

The Program Committee encourages continued improvement in quality of papers and in oral presentations. Careful design of experiments and proper interpretation of results are necessary prerequisites. Strict compliance with the instructions for preparation of abstracts will simplify the task of the Program Committee and will improve the program. Careful editing of abstracts before submission is essential.

Preparation of mimeographed copies of pertinent data is desirable. At least 250 copies should be available for distribution. Material for projection on screens should be used only if mimeographing is impractical.

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

EXTENSION SECTION

Chairman: C. W. Nibler, Department of Dairy Husbandry, University of Nebraska, Lincoln.

Vice-Chairman: M. E. Senger, Department of Animal Industry, North Carolina State College, Raleigh.

Secretary: W. R. Van Sant, Department of Dairy Science, University of Arizona, Tucson.

MANUFACTURING SECTION

Chairman: Stuart Patton, Department of Dairy Science, Pennsylvania State University, University Park.

Vice-Chairman: Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.

Secretary: M. L. Speck, Department of Animal Industry, North Carolina State College, Raleigh

PRODUCTION SECTION

Chairman: G. W. Trimberger, Department of Animal Husbandry, Cornell University, Ithaca, New York.

Vice-Chairman: S. D. Musgrave, Department of Dairying, Oklahoma State University, Stillwater.

Secretary: J. C. Thompson, Ralston Purina Co., St. Louis, Missouri.

NOMINATIONS FOR VICE-PRESIDENT AND DIRECTORS OF A.D.S.A.

D. V. JOSEPHSON, Chairman, Nominating Committee
Department of Dairy Science, Pennsylvania State University, University Park

The By-laws of our Association relating to the nomination of officers were published on page 4 of the October, 1958, issue of the JOURNAL. The By-laws specify that the membership be invited to suggest names of competent individuals from among the members of A.D.S.A. for the consideration of the Nominating Committee.

This Committee feels that it can not do an effective job of preparing a slate of officers without the assistance and full cooperation of the membership. Our Association needs strong, aggressive leadership and much care must be taken in selecting this leadership. Therefore, all members are urged to suggest the names of prominent, competent members who are capable of filling and willing to fill these important offices of our Association.

This year we must elect our Vice-President from the Dairy Manufacturing area. In making your suggestions it should be borne in mind that, if elected Vice-President, your nominee will succeed to the Presidency the following year. For this reason, it will be helpful if your suggested candidate be a man with known administrative capacity and experience. Other factors that might be considered are: service as a Director of A.D.S.A., Section Officer experience, Section or Association Committee leadership, and known devotion to A.D.S.A. objectives.

The two new Directors of the Association for the coming year will be selected as follows: One from the Manufacturing and one from the Production group. Our present group of six Directors includes two from the Extension Section whose terms continue through next year. In accordance with established policy, no one from this latter area will be considered for the office of Director this year.

In recommending candidates for Director, the nominator should consider people who have demonstrated a devoted interest in A.D.S.A. affairs. Members who have held section offices, committee chairmanships, or other Association assignments should be given preference. Known administrative capacity also should be considered.

All suggestions for officers should reach the Chairman of the Nominating Committee by January 1, 1960. The Chairman will assemble the lists and then request a biographical sketch from one of the nominators of each candidate. The Committee will then consider these lists and the supporting information in arriving at the final slate of two candidates for each office, whose names will appear on the election ballot.

The Committee urges the membership to consider this matter seriously. As a Committee of only five men, we are not in a position to recognize all of the qualified people who should be considered for these offices. The future strength and progress of A.D.S.A. will depend upon the leadership we elect. For this reason, we need the help of all members in keeping the offices of the Association filled with the most qualified and devoted people from among our membership.

REPORT OF REPRESENTATIVE TO THE UNITED STATES LIVESTOCK SANITARY ASSOCIATION

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Department of Animal Husbandry, Cornell University, Ithaca, New York

The sixty-second annual meeting of the United States Livestock Sanitary Association was held at Miami Beach, Florida, November 4–7, 1958. Florida was selected as the meeting place this year as a mark of recognition of the growing livestock industry of the South. While the attendance was not as large as at previous meetings, in the Midwest, about 150 persons from all over the country were present. It was a splendid conference, with many excellent papers and committee reports presented.

It was my privilege to be the appointed representative of the American Dairy Science Association at this meeting. While there, the writer served as a member of the Committee on Infectious Diseases of Cattle and on the Committee on Brucellosis at the request of W. D. Knox, a regular committee member who was unable to be present. Both committees presented carefully prepared reports to the general assembly after spending much evening time in discussion prior to their final formulation.

The Infectious Diseases of Cattle Committee emphasized the need for special consideration and study on the problems of genital diseases (with particular attention to nonspecific abortions), anthrax, mastitis, salmonellosis and virus diarrhea or mucosal disease. Special situation reviews included in this committee report covered listeriosis, mucosal disease complex, nocardial mastitis (a newly identified udder disease in California), and vibriosis.

The Brucellosis Committee presented a number of amendments to the present regulations now being observed and followed in the National Brucellosis Program. A plan for nationwide swine brucellosis eradication was prepared. It was believed that with cattle brucellosis now being brought under control and near-eliminated in many states, brucellosis in swine as a source of infection for cattle and man should be vigorously attacked. It was stated that surveys in the United States show about 5% of the hog population infected or suspect, with 2% infected.

ANAPLASMOSIS, A PROBLEM IN MANY STATES

Two papers and a committee report were presented upon the topic of Anaplasmosis. The complement-fixation test was discussed, and treatments with chlortetracycline mixed in cottonseed meal and given orally according to body weight for 60 days have given good results. All cattle treated were negative by complement-fixation test 60 days after treatment and, under field tests on the farm, 83% were negative according to Oklahoma experiments. To save valuable cattle, such treatments, while expensive, are practical.

Anaplasmosis was reported as being diagnosed in sheep in Wyoming, but was believed not to be transmissible to cattle. In California, blood of infected deer was inoculated into 18 calves. Twelve developed the disease within 14 to 28 days later, but none died. Anaplasmosis research is going on in 12 states and at Beltsville. A test-and-slaughter program in Hawaii is giving favorable eradication results under the conditions prevailing there.

LEPTOSPIROSIS COMMITTEE GIVES ADVICE

Leptospirosis in wildlife animals was discussed by H. E. Goldstein of the Ohio Animal Disease Laboratory, and current diagnostic problems were outlined by E. V. Morse of Ames. Surveys in Ohio indicate that of 932 wildlife blood samples tested by the lysis test, deer samples were 20% positive, foxes 25%, raccoons 22%, and skunks 54% positive, 1 to 100 or higher. The point was made that more research is needed relating to the part infected wildlife plays in the spread of this disease in domestic animals.

In discussing leptospirosis tests, Morse stated that leptospirosis positive titers indicate past or present infection. Some cross-reactions may be observed, but they do not become a significant problem. Vaccines may produce reactions for short periods, but usually disappear. Leptospirosis should be considered as a herd problem when a blood test interpretation is being considered.

The Committee on Leptospirosis in its report lists the following points:

1. The test is only an aid in diagnosis. Clinical symptoms should be noted, also.
2. Vaccinate all negative animals and all herd additions.
3. Some vaccinates may abort due to being in the incubation stage at time of vaccination.
4. Vaccinate show-ring cattle prior to the show season.
5. Vaccination applied to infected cattle is of no value.
6. A uniform test is needed.
7. No state program for elimination can be advised until a more specific test has been developed.

SOME OTHER CATTLE DISEASES REVIEWED

Bovine encephalomyelitis, a virus disease of beef and dairy cattle, has been diagnosed in 12 California counties, reported J. B. Enright of Davis. Both sexes may be affected. The disease seldom spreads outside the herd. However, the mode of transmission at present is unknown. Some abortions in beef herds have been noted and an acute form of mastitis in dairy herds is sometimes a symptom. Animals move with a stiff, unsteady gait; they stumble, and hock joints may show swellings. Animals go off-feed, lose weight, and are depressed. Poor condition may persist for a long period and some cattle may die in convulsions, but others recover. Certain antibiotics have been found useful as treatments.

Pustular vulvovaginitis in cattle was reported upon by J. H. Gillespie of Cornell. He said that 15% of the dairy herds in New York, according to his data, have this disease. In one herd of 90 cows in northern New York, 75% were infected. Research indicates that this disease and rhinotracheitis are caused by the same infective agent.

Johne's disease came up for discussion in a paper delivered by A. B. Larson of Auburn, Alabama. Of the johnin tests, he said the intradermal test appears to be the best, according to experiments. It has been found that some adult reactors do become negative later, but the number of recoveries seems to be fewer than with young animals.

The Committee on Vesicular Disease (Foot-and-Mouth, vesicular exanthema, and vesicular stomatitis) reported that 95% of all garbage fed in the United States is now being cooked. All but two states have cooking laws in force.

States were advised to increase funds for garbage inspection, as United States government support is being curtailed for this service.

All cases of vesicular stomatitis should be thoroughly checked, to be certain that none are Foot-and-Mouth disease. Research at Plum Island continues, as does the project on vesicular stomatitis in the coastal region of Georgia, where 79% of the feral swine show antibodies on tests.

PARASITE CONTROL REPORTED UPON

R. C. Bushland from the Kerrville, Texas, Research Station reported upon the work with systemic treatments. Many products by Bayer, Dow Chemical, and American Cyanamid are under investigation. For cattle grubs, neither Bayer nor Dow products give 100% control. They are expensive, too. Treatment for a big bull may be as much as $3. Spray materials are cheaper, it was pointed out. Products to control flies, ticks, and screw-worms, used orally and subcutaneously, also are under investigation at Kerrville.

Some cattle ticks are found in Florida, but are being well controlled by dipping and quarantines. Texas and California also have them along the Mexican border.

The Screw-worm Eradication Program centering at Sebring, Florida, was reported upon by R. S. Sharman of the United States Department of Agriculture. An excellent set of slides was shown of the Sebring operations, in the production and distribution of sterilized male screw-worm flies over Florida, South Georgia, and Southern Alabama. The results to date have been highly successful in the eradication of this animal pest, which has been known in one case to kill a grown steer in ten days.

BOVINE TUBERCULOSIS NOT YET ERADICATED

The Tuberculosis (TB) Eradication Committee made the following recommendations:

1. An increase in cattle testing is needed.
2. The TB reactor tracing service at sales stables and stockyards should be extended.
3. Veterinary colleges should emphasize in their curriculums the importance of TB eradication programs and procedures.
4. More education of cattle owners to combat growing complacency is needed.
5. Maintain rigid quarantines of infected herds.
6. Eradicate TB in other species, especially poultry.
7. Eliminate the no-visible-lesion (NVL) problem through continuing research.

It was stated that 243 counties in the United States are overdue for TB tests.

NEW LEGISLATION, LAWS AND REGULATIONS

The Legislative Committee recommended increased funds for the United States Department of Agriculture for the meat inspection service and for checking the value of new vaccines and biologics. Better control of wild animal imports from abroad was advised.

Uniform state rules and regulations to facilitate interstate livestock movement, and to keep reports of these regulations up to date when amended, were suggested by the Committee on Laws and Regulations.

Officers elected for 1959 are: President, F. G. Buzzell, Augusta, Maine; First Vice-President, James R. Hay, Columbus, Ohio; Second Vice-President, A. P. Schneider, Boise, Idaho; Third Vice-President, W. L. Bendix, Richmond, Virginia; Secretary-Treasurer, R. A. Hendershott, Trenton, New Jersey.

Each year, a complete record of all the papers and committee reports presented at the annual meeting of the Association are published in the *Proceedings*, a neatly printed book of about 350 pages. All members are mailed copies without additional cost.

Membership in the Association is open to any person engaged in livestock sanitary work for federal, provincial, state, territory, county, or municipal governments and to any other person interested in livestock sanitation or milk and meat hygiene.

SOME CURRENT ASPECTS OF ANIMAL DISEASE CONTROL

Report of a Work Party of the Public Health Committee of the American Dairy Science Association for 1959

L. A. BLACK
Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio
AND
W. D. POUNDEN
Ohio Agricultural Experiment Station, Wooster, Ohio

Preliminary reports for 1958 by the National Office of Vital Statistics reveal there were 12% fewer human cases of brucellosis than in 1957. At least part of this accomplishment must be attributed to the national brucellosis eradication program in cattle (5). Recent reports indicate that tuberculosis in cattle is undergoing a slight rise in incidence in some areas of the central states and, because of its relationship to dairy herds, no doubt greater emphasis will be expended on its control. This slight rise in tuberculosis also indicates that more frequent testing may be desired from the viewpoint of public health in respect to sanitary milk control, and that such testing might be done at a 3-yr. interval. Leptospirosis apparently is quite prevalent in many areas and may require considerable effort to prevent its being a recurring problem. Although the organisms are killed by pasteurization, danger may exist to personnel working with infected herds.

Q FEVER

Q fever, a rickettsial disease known to occur throughout the world, has been endemic in cattle, goats, and sheep in certain areas of the western and southwestern states. Preliminary studies and reports by the Rocky Mountain Laboratory of the National Institute of Allergy and Infectious Diseases at Hamilton, Montana, (6), in cooperation with state health department and agricultural agencies, indicate the occurrence of Q fever infections in areas hitherto presumed free from the disease. For example, "We now know that bovine Q fever occurs in every section of the United States. It is especially prevalent in the East, where from 25% to 70% of herds within several states are infected and, undoubtedly, excreting rickettsiae into the environment in milk. We have yet to find a state free of infection! The full implications of all this are not adequately understood, but they are alarming, since the infections which are spreading are the only sources for infection in man. Thus, there is today a need for extensive testing of milk supplies for evidence of bovine infections, to determine the need for local considerations of the disease as a potential hazard to people. Little testing is being done, on milks or human serums, and that being done is experimental studies being sparked from here."

Apparently then, bovine infections with the rickettsiae *Coxiella burnetii* occur in all parts of the United States and are a source of undetermined amounts of human infection and illness. The organisms are excreted in milk and in very large quantities in placental tissues and fluids. The asymptomatic animal infections which are spreading may constitute an increasing public health problem. Human cases of Q fever are associated with casual or occupational exposure to infected livestock and contaminated premises, from residence near infected premises, or from household use of raw milk. Although pasteurization of milk at 145° F. for 30 min. or at 161° F. for 15 sec. destroys the agent (2), an additional 5° higher temperature has been recommended for processing products containing high fat or added sugar. In areas where Q fever has not heretofore been recognized, some dairy products may not receive sufficient heat treatment to destroy such organisms. Where the additional heat treatment recommended by health officials (2) is utilized, this also results in added destruction of the phosphatase enzyme, and thus phosphatase test levels of properly pasteurized products should be well below currently acceptable standards.

STAPHYLOCOCCAL FOOD POISONING

In recent years, outbreaks of disease associated with milk and dairy products have occurred relatively infrequently, ordinarily less than ten per year. Nevertheless, since many people may be involved, these assume great importance. Outbreaks of food poisoning associated with staphylococci in dairy products have been reported in recent years and in 1958 several outbreaks were reported due to natural cheese. In one outbreak, a total of 66 cases was investigated epidemiologically, and bacteriophage types were determined on the coagulase-positive hemolytic staphylococci isolated from cheese. Examination of 24 samples of such cheese showed that all had concentrations of hemolytic staphylococci greater than one million organisms. In another outbreak, hemolytic coagulase-positive staphylococci were isolated from natural Cheddar cheese served to a group of 200 persons who became ill (4).

Although there has been much discussion of staphylococci which are resistant to common antibiotics, the instances when these have been

picked up in association with udders have been surprisingly few (10, 11). However, as long as resistant strains exist there is always the chance that they will be involved in mastitis problems. The finding of preformed staphylococcus enterotoxin in spray-dried milk illustrates the fact that staphylococcus enterotoxin is heat-stable and may be encountered in milk products containing but a few staphylococci if, prior to their destruction by processing, conditions permitted their growth and development of toxin (12). Since such organisms may grow rapidly in pasteurized milk and milk products at 80–120° F., prolonged holding at these temperatures after pasteurization should be avoided.

SALMONELLA

In 1958, an outbreak of *Salmonella dublin* infection was traced to certified raw milk, with 11 laboratory-proved and 19 suspect cases. Three isolations of Salmonellae organisms were made from cows in the herd of about 400 animals (3). In this connection, it may be of interest to note that a working party of the Public Health Laboratory Services in England has found Salmonella present in a wide variety of raw, processed, and complete feeding meals, especially imported bone, bone products, and fish meal. Although viable counts were low (less than 100 per 100 g.), 88 serotypes were isolated, and further work was planned as to their epidemiological importance (9).

YEASTS, ACTINOMYCETES, AND VIRUSES

Udder infections due to yeasts and yeastlike fungi, such as Cryptococcus (8), and actinomycetes such as Nocardia (7), can present some potential problems of human involvement. The aerobic actinomycetes which are pathogenic for humans have been isolated from mastitis in cattle in various sections of this country. Their biology is not very well understood; virulence differs, so that infections may vary from almost inapparent illness to severe mastitis, and some strains of the organism show a marked resistance to heat as isolated from the milk of infected cows. The organisms are resistant to most antibiotics and their use is more likely to aggravate than to help the problem. The finding of this organism in the udders of dairy cattle should be of considerable public health importance and requires further study.

The isolation of members of the lymphogranuloma venerum group of viruses from humans and almost all kinds of livestock, including cattle, indicates the tremendously broad areas in which these infections are involved. Perhaps of some significance is the finding that some of these viruses can exist in udders (1) and can be shed from them.

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ABSTRACTS OF PAPERS

PRESENTED AT THE WESTERN DIVISION MEETING

UNIVERSITY OF ARIZONA, TUCSON
JULY 13 TO 15, 1959

Mastitis Studies in a Dairy Herd. GAYLE GILDA AND E. GRANT MOODY, Arizona State University, Tempe.

A 7-yr. study of 254 separate incidents of mastitis in 87 cows in the Arizona State University herd was made. An attack of mastitis resulted in the immediate loss of more than 10 lb. of milk. The decrease in production occurred 24 hr. before the appearance of clinical symptoms. The incidence of mastitis in a herd was drastically reduced by proper management and the culling of cows with chronic mastitis. Bacteriological analysis of secretions from 15 cows indicated that *S. aureus*, the most commonly occurring organism, showed a measure of in vitro sensitivity to all antibiotics tried except polymixin.

Dried Molasses Beet Pulp vs. Chopped Alfalfa Hay as a Preservative for Oat and Vetch Silage. D. D. BULLIS, R. H. KLIEWER, AND I. R. JONES, Oregon State College, Corvallis.

Twenty-four Holstein and Jersey cows, paired according to breed, age, body weight, production, stage of lactation, and gestation were fed oat and vetch silage to appetite, preserved with 200 lb. of dried molasses beet pulp or chopped alfalfa hay. No significant difference ($P < 0.05$) in FCM, body weight change, or FCM produced per pound of dry matter consumed was observed. However, there was a significant ($P > 0.01$) difference in silage dry matter consumed per day per cow and a significant difference ($P > 0.02$) in silage dry matter consumption per pound of body weight in favor of the molasses beet pulp preserved silage.

There was less plant juice loss, less top spoilage and, therefore, less total dry matter loss with the dried molasses beet pulp preserved silage than with the chopped alfalfa hay preserved silage. The 7,500 lb. greater juice loss with the chopped alfalfa hay silage may be conservatively estimated to contain 8% dry matter, or an apparent saving of 600 lb. of dry matter in favor of the dried molasses beet pulp preserved silage.

Ensiled forage preserved with chopped alfalfa hay was found to be higher in protein, fiber, fat, and ash than the same forage preserved with molasses dried beet pulp. However, the latter silage had the highest N.F.E. and TDN values.

Comparative Feeding Value of Oat and Pea Silage Preserved with Dried Molasses Beet Pulp vs. Ryegrass Screenings. D. D. BULLIS, R. H. KLIEWER, AND I. R. JONES, Oregon State College, Corvallis.

The feeding value of high-moisture oat and pea forage preserved with ryegrass screenings or dried molasses beet pulp at the rate of 200 lb. per ton was compared, using 22 Holstein and Jersey cows paired into two groups and fed to appetite. A 4-wk. Latin-square crossover experimental design was used with a 4-wk. preliminary period and a 1-wk. change-over period between treatments.

Cows in each group consumed significantly more ($P < 0.01$) molasses beet pulp preserved silage (13.20 lb. more per day per cow), but failed to show a significant increase ($P > 0.05$) in energy-corrected-milk (ECM) compared to the rygrass screenings preserved silage. However, a significant increase ($P < 0.05$) in body weight for those animals fed the molasses beet pulp preserved silage was shown, reflecting the increase in consumption.

Effect of Feeding Low Levels of Chlortetracycline upon the Health and Milk Production of Dairy Cows. R. H. ROSS, D. L. FOURT, J. W. BAILEY, AND D. L. THACKER, University of Idaho, Moscow.

Twelve Holstein cows paired into two groups were used to determine the value of feeding 0.1 mg. chlortetracycline per pound body weight upon milk production, mastitis, bloat, and footrot. A reversal trial consisting of four 4-wk. periods was used. An additional 48 cows were used to study the effect of chlortetracycline upon mastitis, bloat, and footrot.

Feeding chlortetracycline had no effect upon milk production, mastitis, bloat, or footrot. Milk production was 37.5 lb. and 37.7 lb. 4% FCM for the control and experimental groups, respectively. There was one case of footrot in the control group. There were five cases of bloat in each group. Chronic cases of mastitis continued in both groups and no new cases developed in either group.

Alfalfa "Hayfers" vs. Baled Alfalfa Hay for Milk and Butterfat Production. R. H. ROSS, D. L. FOURT, AND F. C. GEPHART, University of Idaho, Moscow.

Twelve Jersey cows paired into two groups were used to compare baled alfalfa hay and "hayfers." The alfalfa hayfers were made by compressing chopped alfalfa hay into wafers about 4 in. in diameter and 1 in. thick. One cutting of hay from the same field was used

for both lots of hay. No supplemental feeds were used except iodized salt and bone meal fed free-choice. Per cent butterfat in the milk was determined on three-day composite tests taken continuously. A reversal trial was used, consisting of two, 5-wk. periods.

Mean daily milk production was 19.9 lb. 4% FCM for both groups. There was a significant reduction in butterfat test ($P < .05$). Baled hay averaged 5.74% and hayfers 5.48% butterfat, a reduction of 0.26%. The cows while receiving hayfers ate 27.7 lb. and wasted 1.6 lb. hay per cow per day and gained 0.74 lb. body weight per cow per day. While receiving baled hay, they ate 22.0 lb. hay and wasted 7.0 lb. per cow per day and lost 1.04 lb. body weight per cow per day. These differences were significant ($P < .05$).

Milk and Butterfat Production from Mash and Pelleted Grain Rations. LELAND TIGGES AND G. M. WARD, Colorado State University, Fort Collins.

The same ingredients were prepared as a coarsely ground grain mix and as 3/8-in. pellets and fed to 27 purebred Holstein-Friesian cows in the Colorado State University herd. The grain was fed to each group at the rate of approximately 1 lb. per 4 lb. of 4% FCM. Alfalfa hay and corn silage was group-fed in outdoor mangers. All cows were milked in a six-cow elevated milking parlor.

The study contained four trials consisting of a ten-day preliminary period followed by a 20-day experimental period. The order of trials was (I) pellets, (II) mash, (III) pellets, and (IV) mash. The average daily milk production and butterfat percentage for each period was, respectively: (I) 43.7, 3.39; (II) 42.4, 3.59; (III) 40.8, 3.31, and (IV) 38.6, 3.34. Differences between the two rations in milk production, fat test, and 4% FCM were not statistically significant, although the lower fat test obtained with the pelleted grain approached significance at the 5% level. All cows were able to finish eating their allotted grain in either form during an average of 7 min. which they spent in the milking parlor.

A Summary of Three Years' Work with Holstein Steers. R. O. RUPP, G. M. WARD, MELVIN H. HAZALEUS, AND H. B. LINDHOLM, Colorado State University, Fort Collins.

During the past 3 yr., over 100 Holstein steers were raised on various feeding systems and marketed at different ages and weights. From this study, the following conclusions can be drawn:

1. Veal production from dairy calves generally is not profitable in Colorado.
2. Cattle raised to an age and weight of feeders (500-700 lb.) were generally more profitable than the same animals fed to 1,000 lb.
3. Steers fed on straight roughage or roughage plus a limited amount of grain will make rapid gains and yield carcasses generally grading U.S. Standard.
4. All roughage rations require a 50- to 60-day longer feeding period to produce the same weights and carcass grades as steers fed grain at the rate of about 10 lb. per day.
5. All roughage feeding resulted in the lowest costs per pound of gain.
6. Dried beet pulp can replace 90% of the roughage of a normal hay and grain ration for a period of 130 days. This system gave the fastest gains and the highest-grading carcasses.
7. Stilbestrol or hormone implants increased gains and feed efficiency comparable to values reported for beef breeds.

Animal Fat as a Replacement for Barley in Dairy Cattle Rations. J. F. STEIN, M. A. WADE, D. W. CASSARD, AND L. WALKER, University of Nevada, Reno.

Sixteen lactating Holstein cows were divided into four comparable groups. Two concentrate mixtures were fed. The experimental mixture contained approximately 10% stabilized animal fat and was formulated on the basis of substituting 1 lb. fat for 2.5 lb. of barley in the control mixture.

In a change-over design, each group remained on each of the following treatments for a period of 5 wk. with a seven-day transition period between treatments: (I) Control fed at the rate of 1:6 FCM; (II) fat-concentrate at 1:6 FCM; (III) fat-concentrate at 1:8 FCM; (IV) fat-concentrate pelleted and fed at 1:8 FCM. Adjustments were made in the rate of feeding at the beginning and midway in each treatment period. Each group received good-quality alfalfa hay ad libitum under dry-lot feeding conditions. The mean daily pounds fat intake from concentrates (D.M. basis) were 0.14, 0.60, 0.45, and 0.50, respectively.

Mean daily pounds FCM produced on the various treatments were (I) 35.4, (II) 38.5, (III) 36.7, and (IV) 37.5. Feeding the fat-concentrate (II) resulted in a significant increase ($P < 0.01$) in FCM production over (I). No significant differences in body weight of animals were noted between treatments.

Feeding the fat-concentrate (II), (III), (IV) resulted in a significant increase in plasma lipid value ($P < 0.01$). No significant differences in plasma glucose and ketone bodies were observed. These values were in the normal range as reported in the literature.

Pineapple Plant as a Feed for Dairy Cows. K. K. OTAGAKI AND K. MORITA, University of Hawaii, Honolulu.

There is a lack of forage for milking cows supplying the Honolulu milk market because

of lack of agricultural areas for economical forage production. Pineapple plants are readily available in tremendous quantities, but only meager information is available on their nutritive value. Twelve Holstein-Friesian milking cows were allotted to three treatments in a double change-over design with 5-wk. periods. All cows received the same basal ration according to size and production and were supplemented with chopped Napier grass, ensiled or fresh pineapple plant to the limit of appetite. There were no significant differences between treatments in actual or fat-corrected milk production. Highly significant differences were noted in butterfat tests between cows within groups and between treatments. Some of these differences may be attributed to significantly lower dry matter intakes on fresh and ensiled pineapple plant regimes. There were no significant differences in milk flavors due to treatments. There were no indications of any adverse physiological effect from feeding pineapple plants as a source of roughage to lactating cows. Digestibility studies of fresh pineapple plant showed a high degree of digestibility of the crude fiber fraction. The DCP and TDN values for fresh pineapple plant were 6.8 and 74.8%, respectively, on the oven-dry basis.

Relationship of Bacteria and Progesterone to the Androgenic Activity of Cow Feces. C. E. LUEKER, University of California, Davis.

An investigation was designed to study the paradoxical fact that the feces of the female bovine contain a greater androgenic activity than bull feces. In the course of this experiment, the possibility of bacterial action being responsible for the androgenic property of incubated cow feces was studied. It was shown by the chick comb growth assay method that no androgenic response was obtained when cow feces were sterilized before they were incubated. However, when cow feces were sterilized, inoculated with fresh cow feces and then incubated, an androgenic response was obtained. The androgenic property found in incubated feces from female cattle appears to be the result of the conversion of a nonandrogenic material to an androgenic substance. Addition of progesterone to cow feces before incubation increased the androgenic activity. Thus, the conversion of progesterone to an androgenic substance by bacterial action provides a plausible explanation for the presence of androgenic activity found in cow feces.

Feeding Value and in Vitro Performance of Live Yeast Cultures. HERBERT JORDAN AND G. M. WARD, Colorado State University, Fort Collins.

A live culture yeast supplement added at the rate of 40 lb. per ton of grain was fed to eight Holstein cows and compared to a control ration of the same grain mix without the supplement. Data were recorded for five periods of 2 wk. each: (I) control, (II) yeast, (III) control, (IV) yeast, (V) control. The average production of 4% FCM and the average butterfat percentage for each period was (I) 53.3, 3.8, (II) 51.7, 3.8, (III) 53.8, 3.9, (IV) 54.0, 3.8, (V) 49.7, 3.9. The yeast-supplemented feed was not as readily consumed for the first few days after changing feeds.

A live yeast culture produced by another manufacturer was used to evaluate gas production in an artificial rumen apparatus. Alfalfa hay, alfalfa and grain, and purified cellulose (Solka-Floc) were added to strained rumen fluid. Gas production was measured on each of these substances alone and with the addition of yeast culture at the rate of 2% of the feed substrates. The yeast culture increased gas production from the purified cellulose, but had no consistent effect upon the other substrates.

Penicillin Levels in Milk from Lactating Cows Fed Various Amounts of Procaine Penicillin. S. R. SKAGGS AND D. D. MILLER, New Mexico State University, University Park.

The United States Food and Drug Administration had warned farmers that continued contamination of milk with antibiotics would bring disciplinary action by the U.S.F.D.A. Since penicillin salt had been advertised as a safeguard against bloat in cattle, it appeared that a study should be made as to the possibility that cows that received penicillin orally transmitted significant amounts to their milk.

Two lactating dairy cows were fed daily doses of procaine penicillin in salt sprinkled over their concentrate feed. Milk samples from each cow were analyzed for penicillin content by the Bacto-Concentration Disk method. No penicillin was found in the milk when the cows received 88.9 mg. (86,700 I.U.) penicillin daily. When the feeding level was increased to 177.8 mg. daily, approximately 0.05 I.U. penicillin per milliliter was found in the milk. A feeding level of 277.8 mg. daily increased the milk level to between 0.10 and 0.15 I.U. per ml. Blood samples from these cows showed approximately the same level of penicillin content as the milk samples.

A Method for Getting More Value from DHIA Records. C. J. HOFFMAN, Colorado State University, Fort Collins.

A booklet has been prepared which dairymen can use to chart and graph data from their Dairy Herd Improvement Association (DHIA) records. These charts and graphs help to determine weaknesses in herd management.

The charts help dairymen to observe the monthly production of a herd, calving interval, percentage of dry cows, number of cows removed from the herd and the reasons why, age of heifers at freshening, average age of cows in the herd, distribution of production by cows, and the monthly feed-cost chart.

Workshop sessions have been held with 16 DHIA. All have been highly successful and it has been suggested by dairymen that this be an annual program. A number of other states have learned about Colorado's approach and are in the process of developing similar programs.

Effect of Ration Fiber upon Respiration Rate and Rectal Temperature. MELCHIOR CADENA, C. AND J. V. BATEMAN, Inter-American Institute of Agricultural Sciences, Turrialba, Costa Rica.

Four daughters each, of three different milking Criollo bulls, were submitted to 35° C. and 25-mm. vapor pressure in a climatic chamber while on diets containing 16, 23, and 25.5% fiber. The effect of these fiber levels on respiration rates and rectal temperatures was studied. The correlations between hematocrit and respiration rates, and hematocrit and rectal temperatures, were determined. They were −.51 and −.74, respectively. The effect of fiber level on respiration rate was not significant, but the difference in response of different animals was highly significant. Fiber level had a significant effect upon rectal temperature. The responses of individuals were also highly significant. These results led to the following conclusions: 1) The response to heat stress with different levels of ration fiber varies with individuals. 2) Rectal temperatures change with levels of fiber; therefore, experiments relating to physiology and thermal stress should take this into account. 3) The fiber content of the ration should be reported.

Controlling Oxidized Flavor with Heat-Treated Milk. D. D. MILLER AND S. R. SKAGGS, New Mexico State University, University Park.

Theoretically, the sulfhydryl groups produced by heating milk should act as reducing agents and, thereby, inhibit the production of oxidized flavor. This work was done to determine the validity of the above theory.

Milk, which produced oxidized flavor spontaneously, had various amounts of the same milk heated to 90° C. for 45 min. added to the raw milk. The samples were pasteurized, cooled, and stored for seven days at 4° C. Daily organoleptic tests were made.

Inhibition of oxidized flavor was noted at the 2% level. Cooked flavor developed at the 3.5% level. Greatest inhibition, before cooked flavor developed, was observed at the 3% level. Complete prevention of oxidized flavor occurred in some samples at the 3% level, but this was dependent upon the susceptibility of the milk to oxidized flavor. When 0.1 p.p.m. copper was added to the samples, complete prevention never occurred, but good inhibition was noted at the 3% level. Heating the milk for shorter or longer periods was not as effective as 45 min. at 90° C.

Influence of Brand of Cocoa on Consumer Acceptance of Chocolate Drink. H. S. WILLARD, W. R. THOMAS, AND C. E. BERNBECK, University of Wyoming, Laramie.

To determine if brand of cocoa alters consumer acceptance of chocolate drink, between 20 and 50 college students of both sexes were given three samples successively of chocolate drink per session and were asked to express degree of likes and dislikes on a Hedonic scale. Each drink was identical except for the brand of cocoa. Fifteen series of three samples were submitted, with one brand of cocoa common to each series being used as control. Differences from the mean score of the control sample showed that consumer preference was not influenced by the per cent of fat or method of manufacture of the cocoa. Control samples appearing in different series, as a rule received somewhat the same score. On an average, processed cocoas out-scored the natural cocoas, whereas the high-fat cocoas out-scored the low-fat cocoas; but differences were of no statistical significance.

The Consumer Acceptance of Milk Beverages as Affected by Fat and Solids-not-fat Content. J. W. STULL AND J. S. HILLMAN, University of Arizona, Tucson.

A consumer-preference study was conducted to provide statistically reliable data on which standards and practices relating to the optimum composition of milk beverages can be based.

The addition of solids-not-fat (SNF) to milk beverages is not provided for in most standards. There is a general awareness, however, that the addition of from 0.5 to 2.0% SNF to either whole, low-fat, or nonfat milk beverages improves palatability, flavor, food value, and utilization.

Taste discrimination or threshold tests involving 2,132 paired and triangulation decisions were conducted with adult men and women who were not trained dairy products judges. The results showed that people could consistently differentiate between milk beverages with variations in fat and SNF contents of 0.5 and 1.0%, respectively.

Paired preference comparisons within and between each beverage class (whole, low-fat, and nonfat beverages) were made with approximately 10,000 persons in retail food markets, public gatherings, and public schools. Beverages with 0.5 to 1.0% added SNF were preferred. There was no significant difference in preference for a regular whole milk (3.5% fat, 8.5% SNF) when compared with a low-fat beverage (2.0% fat, 10% SNF).

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BOOK REVIEWS

METABOLISM IN THE RUMEN. E. F. Annison and Dyfed Lewis. John Wiley & Sons, Inc., N. Y. 184 pp. 1959.

This small volume, the latest addition to the series of Meuthuen's monographs on biochemical subjects, will be welcomed by ruminant nutritionists as the first published work devoted entirely to rumen metabolism. It should also be of interest to biochemists and microbiologists in related areas, because of the unique biological organization of the rumen.

It is regrettable in one sense that a more comprehensive volume was not undertaken, in which rumen physiology and the broad field of ruminant nutrition also were encompassed. However, in confining their coverage to metabolic activities of the rumen, the authors have achieved their objectives extremely well, and the book will provide a valuable reference aid both in teaching and in research.

The book is organized into six chapters dealing with various aspects of metabolism in the rumen. Chapter 2, on the microbiology of the rumen, provides an excellent summary of the types and characteristics of microorganisms found in the rumen, together with a discussion of the biological parameters of the rumen that exert control on the microbial population. This chapter should be especially valuable to the many students and investigators of ruminant nutrition whose primary interests are in areas other than bacteriology. The greater part of the text deals with the metabolism of carbohydrates and nitrogen in the rumen. Unquestionably, this is where the emphasis belongs. An entire chapter is devoted to absorption from the rumen, with subdivisions dealing with the absorption of volatile fatty acids, ammonia, glucose and lactic acid, gases, and inorganic ions and water. A discussion of techniques used in absorption studies and difficulties in measuring absorption rates also is included. The concluding chapter deals with ruminant disorders associated with metabolic disturbances in the rumen and, although none of the disorders are discussed comprehensively, sufficient data are presented to adequately link the particular disorder to some rumen metabolic activity.

This book will make a valuable supplemental text for courses in animal nutrition and, while it is limited in scope, the price is modest.

R. E. BROWN
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ELEMENTS OF DAIRYING LABORATORY MANUAL. E. E. Bartley, F. E. Eldridge, E. L. Farmer, F. C. Fontaine, and G. B. Marion. Burgess Publishing Company, Minneapolis, Minnesota. 2nd ed. 1959.

This laboratory manual was prepared for use in the college introductory course in dairying. In it, 11 exercises of the type that would permit maximum student participation are outlined.

The subject matter areas covered and the number of exercises devoted to each include: Composition of Milk, 2; Tour of the Dairy Processing Plant, 1; Quality of Dairy Products, 1; Feeding Dairy Cows, 3; Management and Housing, 1; Milking Management, 1; Artificial Breeding, 1; and Dairy Cattle Judging, 1.

The exercises are well illustrated with pictures and schematic drawings, along with procedures and problems.

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THE FREEZING POINT OF MILK. A REVIEW[1]

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SUMMARY

The literature clearly shows that the freezing point of milk varies within relatively narrow limits. Some of the variations that do occur have been attributed to seasonal effects, feed, water intake, time of day (i.e., morning versus evening milk), breed of cow, and method of handling samples. In handling milk samples, it has been reported that storing them at low temperatures, or freezing them, may raise the freezing point. Some analysts claim that heat treatment affects the freezing point slightly, whereas other workers have not observed any change. Vacuum treatment of milk has been reported to raise the freezing point slightly. Some of the apparent differences in freezing points reported by different workers may be due to differences in analytical techniques. Comparison of the freezing points reported by different analysts for identical samples reveals a need for more careful standardization of the method. It is believed that more attention should be given to the calibration of the thermal sensing element. It has been suggested that salt solutions or saturated aqueous solutions of stable compounds should be used as calibration standards instead of sucrose solutions. The necessity of standardizing the manner in which the thermometer is tapped has been emphasized. The ease and speed of determining freezing points with the recently designed cryoscopes, employing thermistors as thermal sensing elements, should increase the use of cryoscopes for routine analysis. The reported results show that the freezing point of milk is a useful index for detecting added water. However, the decision to classify a sample as watered must be based upon careful consideration of all of the available evidence. One should not rely on the conclusions of others without examining their data. The published evidence indicates that the present official standard in this country should be re-examined. It has been suggested that the adoption of a minimum freezing point standard might overcome the limitations of using an assumed average as a standard.

In the past 10 yr., interest in the freezing point of milk has been stimulated by the publication of a number of papers concerning cryoscopic methods and standards. The results reported in these papers were believed by some to contradict previously published results; whereas, actually, most of the recent results could have been predicted on the basis of earlier literature. As is too often the case, interpretations or conclusions are handed down from generation to generation, but the facts on which they were based are overlooked or forgotten. An attempt will be made in this review to re-examine the conclusions in the light of the published facts.

THEORETICAL CONSIDERATIONS

The freezing point of cow's milk is the least variable property of milk and, consequently, it is of interest from both a practical and theoretical standpoint. Its practical value is that it provides the most accurate means of detecting added water. It is of theoretical interest because of the physiological questions which it raises. For example, by what mechanism is the freezing point held within such narrow limits? The classical answer is that milk is in osmotic equilibrium with

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[1] Reprints available for 90 days after publication of this paper. For prices see page 1434 of the August issue.

the blood. (Incidentally, the freezing point is an indirect measure of the osmotic pressure.) The findings of van der Laan (100), Kern (54), and Koenig (58) support this answer. However, it does not explain the reason for the narrow range in osmotic pressure of the blood.

The osmotic pressure and, hence, the freezing point of milk or of any aqueous system is dependent on the concentration of water-soluble constituents. The addition of a solute to any solvent depresses the freezing point. The mathematical relationship between the freezing point depression and concentration of solute was worked out by Raoult (75) and is expressed in the following formula: $T_f = K_f m$, where T_f is the freezing point depression, K_f the molal depression constant, and m the molal concentration of the solute. The molal depression constant differs for each solvent. In water, it is 1.86° C. As Raoult pointed out, this exact relationship is valid only for dilute solutions of undissociated solutes.

According to Cole and coworkers (21, 22), lactose and chlorides account for almost 75% of the total freezing point depression. They observed a slight difference between Jersey and Friesian milk. Their results indicate that over 50% of the depression is due to lactose and about 25% is due to chlorides. The fat and insoluble proteins do not have any measurable effect. Dharmarajan *et al.* (30) claimed that lactose and chlorides accounted for about 77% of the freezing point depression of cows' milk and about 72% for buffalo milk.

Pinkerton and Peters (70, 73) also studied the contribution of lactose to the freezing point depression and concluded that it was the most important factor. Rees (76), in a study of the variation of freezing points, stated that changes in the concentration of the nonchloride ash fraction were the primary cause of variations. The principal variable in this fraction was believed to be the soluble acid phosphates.

Numerous workers (14, 21, 22, 23, 50, 70, 72, 73) have pointed out the complementary nature of the concentration of lactose and chloride. In general, as the concentration of lactose decreases, the chloride concentration increases, and vice versa. The fact that the freezing point is less variable than other properties of milk has been attributed at least in part to this complementary relationship. However, Aschaffenburg and Rowland (6) noted a case where this complementary relationship did not exist. They found that the lactose-chloride ratio of morning versus evening milk did not change, even though there was a change in the freezing point. They stated that evening milk appeared to be naturally watered morning milk.

CRYOSCOPIC METHODS

In 1921, Hortvet (45) published a new method for determining the freezing point because he felt that earlier methods were not sufficiently standardized. Since there is no published proof that earlier methods gave data comparable to Hortvet's data, this review will deal only with the literature pertaining to the Hortvet method, or methods reported to give results comparable to this method.

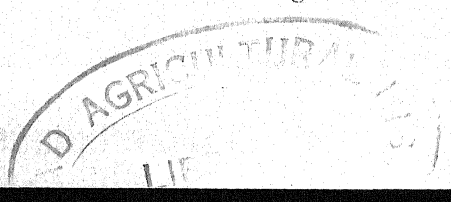
Hortvet recognized the empirical nature of cryoscopic procedures and, therefore, he emphasized the need for using standardized equipment and technique. To

minimize variations between analysts, he gave a detailed description of the apparatus and procedure. As an additional device to compensate for variability between analysts, his procedure involves the measurement of the difference between the freezing point of a standard solution (i.e., 7% sucrose) and the freezing point of milk. Theoretically, any systematic errors should be reflected in both observed values and should offset each other. This feature of the Hortvet technique places a special emphasis on the calibration procedure that is apparently not recognized by all analysts. Of course, the calibration procedure will compensate for systematic errors only when variations in technique have exactly the same effect on the observed results for both the standard solution and milk. Hortvet's choice of sucrose solutions as calibration standards may have been unfortunate, since the freezing characteristics of sucrose solutions and milk are different, according to Shipe (83). This observation suggests that variations in technique may not have exactly the same effect on the standards as on the milk and, hence, compensating effects are reduced. Furthermore, it is difficult to obtain reproducible results with sucrose solutions and they readily undergo microbial decomposition.

To overcome the difficulties encountered with sucrose calibrations, Aschaffenburg and coworkers (4, 5, 7) have recommended that the thermometers should be calibrated by a recognized testing institution such as the British National Physical Laboratory. Sutton and Markland (95, 96) have questioned the wisdom of such a procedure and have presented data indicating that the sucrose calibrations give better results. Some analysts have reported that salt solutions give more reproducible results than sucrose solutions. Shipe (84) has suggested the use of stable compounds whose solubilities in water are such that saturated aqueous solutions of these compounds would have freezing points in the desired range. It is this author's opinion that Hortvet's idea of determining the difference between the freezing point of a standard and milk is basically sound.

Attempts have been made to apply a correction factor to the observed Hortvet freezing point, to compensate for the deviation from the true freezing point. Monier-Williams (68) and Stubbs (89–92) have reported on their efforts to determine the magnitude of this deviation. Elsdon and Walker (37) have given an excellent review of the factors involved. However, from a practical viewpoint it is not necessary to determine the true freezing point. The key problem lies in obtaining reproducible results between samples and analysts.

Horvet (45) assumed that reproducible results could be obtained by his method. However, he did not report any evidence to prove this assumption. It is generally assumed that a given analyst can obtain reproducible results. On the other hand, there is reason to question the assumption that different analysts will obtain comparable results. A report by Shipe, Dahlberg, and Herrington (85) revealed considerable differences between the observed results for nine collaborating laboratories. For one series of seven samples, there was a maximum variation of 0.0275° C. between the average freezing point values. However, in most cases, a given laboratory deviated from the average for each sample by



about the same amount. This indicated that each analyst was able to reproduce his own work, but his values were not necessarily comparable to those of other analysts. A collaborative study by Robertson (79), involving 19 laboratories, revealed similar results. The collaborators were supplied with three milk samples, two of which were identical; the third sample contained 1.5% added water. With the exception of five laboratories, they were able to correctly differentiate between samples. However, there were considerable variations in the observed freezing point values. Additional evidence of the variations between laboratories is reported in a collaborative study by Shipe (83).

In contrast to the variable results reported, Elsdon and Stubbs (35) reported good agreement between six laboratories on one sample of milk. Using a procedure prescribed by Aschaffenburg and King (5), seven laboratories were able to obtain comparable results. In this case, the analysts used an 8.5% sucrose solution and, therefore, knew the expected results in advance. Nevertheless, these two reports indicate that good agreement can be obtained between analysts with the Hortvet method. However, numerous workers have observed causes for lack of reproducibility and have in some cases suggested modifications.

Elsdon and Stubbs (33) observed that the thermometer calibrations varied with the storage temperature and age of thermometers. Jones (52) observed that leaving the starter tube in the sample during the freezing affected the observed freezing point of water. Monier-Williams (68) suggested that the alcohol be removed from the tube surrounding the sample tube at the time of seeding, so as to minimize heat transfer during the freezing process. Stubbs (91) made a similar recommendation. Stubbs and Elsdon (94) made specific recommendations for standardizing thermometers.

Temple (99) designed a modified cryoscope in which a mechanically refrigerated bath was substituted for Hortvet's ether-cooling system. This cryoscope was easier and it permitted better control of the bath temperature. Temple's cryoscope has been used extensively in England.

Sutton and coworkers (97) have studied some of the potential sources of errors in the Hortvet procedure. They pointed out that due to the slow adjustment of glass to changes in temperature the zero point of the thermometer might change as much as 0.005° C. during a day. They also noted that the dimensions of the freezing tube and thermometer bulk were critical. They stated that it is essential to limit the amount of stirring after seeding. They suggest modifying the Hortvet apparatus so that the alcohol surrounding the sample tube can be removed at the time of seeding.

Shipe, Dahlberg, and Herrington (85) have designed a modified cryoscope which employs a mechanically refrigerated bath. Their cryoscope also has mechanical devices for stirring the sample and for tapping the thermometer. They observed that mechanical tapping markedly reduced variations between analysts. This observation has been confirmed by Dubin (32). Green (40) has designed a cryoscope with a mechanical stirrer and tapper. He has also observed better results using these mechanical features. Incidentally, Monier-Williams (67) in 1915 had used a mechanical tapping device.

Gaballah (39) designed a cryoscope with four freezing tubes. His apparatus is mechanically refrigerated and the sample is stirred mechanically. He claimed that four determinations could be made in 15 min. Knowles (57) modified an electric water cooler to provide a bath for a cryoscope.

Bowman, Trantham, and Caulfield (16) developed a cryoscope, using a thermistor as the thermal sensing element in place of a mercury in glass thermometer. The American Instrument Company, Inc.[1] and Fiske Associates, Inc.[2] have manufactured cryoscopes which use thermistors. Results obtained with the Fiske cryoscopes have been reported by Hileman and Wiggin (42) and Shipe (82, 83). The results indicate that determinations can be made easily and rapidly (20 to 30 per hour) with this cryoscope. Shipe's data indicate that it is possible to obtain results with the Fiske that are comparable to the Hortvet. However, the results indicate that a more standardized procedure is necessary to reduce variations between analysts.

The British Standards Institution (17) has just published a revised set of directions and specifications for the Hortvet method. This publication contains a very detailed description of the apparatus and the procedure which overcomes some of the ambiguities of Hortvet's directions. However, the new procedure for tapping the thermometer seems to be unnecessarily complicated. This procedure was first recommended by Aschaffenburg and King (5). Shipe (84), by using a mechanical tapper which minimizes the human factor, obtained the same results as with the more complicated British method.

FREEZING POINTS OF COW'S MILK

In 1923, the Association of Official Agricultural Chemists (48) officially adopted the Hortvet cryoscopic method. In connection with the adoption of the method, they recommended that −0.550° C. be considered the average freezing point of normal milk, with the allowance of a 3% tolerance. These recommendations were based on the observations of Hortvet (45, 46) and a collaborative study conducted by Bailey (12). Libby, McNeill, and Libby, Chicago and Morrison, Illinois, the Connecticut Agricultural Experiment Station, and the Minnesota Agricultural Experiment Station participated in this study. The results are shown in Tables 1 and 2.

As will be noted in Table 1, the average freezing points of 179 individual cow samples and 61 herd samples were −0.545 and −0.544° C., respectively. It should be noted that there are appreciable differences between the averages obtained by the three laboratories. For example, the herd average for Libby, McNeill, and Libby laboratory was −0.542°, whereas the corresponding value for the Minnesota samples was −0.551°. This difference is equivalent to about 1.5% added water. This difference between laboratories might be interpreted as indicating that the milk supply was different in the two areas, or that the tech-

[1] American Instrument Co., Inc., Silver Spring, Maryland.
[2] Fiske Associates, Inc., Hathorne, Massachusetts.

TABLE 1
Summary of freezing point data reported by Bailey (12)

Laboratory	No. of samples	Maximum ° C.	Minimum ° C.	Average ° C.
		Individual cow samples		
Minnesota	77	−0.562	−0.534	−0.547
Libby, McNeill, & Libby	27	−0.563	−0.532	−0.546
Connecticut	75	−0.566	−0.530	−0.543
	179	−0.566	−0.530	−0.545
		Herd samples		
Minnesota	15	−0.562	−0.545	−0.551
Libby, McNeill, & Libby	37	−0.553	−0.530	−0.542
Connecticut	9	−0.560	−0.535	−0.544
	61	−0.562	−0.530	−0.544

TABLE 2
Dates of collection of freezing-point samples as reported by Hortvet (45) and Bailey (12)

Laboratory	No. of samples	Dates collected
	Individual cow samples	
Minnesota	15	May
	25	June
	1	August
	19	September
	17	Dates not reported
Libby, McNeill, & Libby	27	September (14 to 23)
Connecticut	73	April 5 to May 26
	2	July 7
	Herd samples	
Minnesota	15	Dates not reported
Libby, McNeill, & Libby	2	March 29
	3	April 1
	5	June
	4	July
	2	August
	21	September
Connecticut	9	April 13 to May 2

niques used by the different laboratories were different, or both. No attempt had been made to check the reproducibility between laboratories. Of course, the differences may have been due to the limited number of samples analyzed.

In addition to the number of samples being limited, the period of collections was limited. As is shown in Table 2, there were no samples reported as having been collected during October, November, December, January, or February. This failure to collect samples in the winter months apparently was due to the assumption that the season of the year has no effect on the freezing point.

The fact that Bailey's collaborative study revealed differences between the freezing point of morning and evening milk generally has been overlooked. Of the samples reported, 49 were identified as evening samples and 44 as morning samples, with average freezing points of −0.542 and 0.555° C., respectively.

It appears that the results and limitations of this initial work with the Hortvet method have received less attention than the following statement of Hortvet (45)

that: "Milk, freshly drawn, from any variety of cow whether of high or low breed, from whatever region of the country, from animals in stable, or in pasture, whether poorly or substantially fed, whether drawn during a period near or remote from parturition, in winter or in summer, morning or evening, or whether the yield be scant or abundant, has a freezing point which varies but little around −0.550° C., although under various influences the chemical composition changes in enormous proportions." This statement has been accepted by many as meaning that the freezing point is a constant, in spite of the qualifying phrase, "which varies but little around −0.550° C." The statement does imply that the freezing point is independent of environmental factors. This implication has been widely accepted, although their data do not substantiate it. In fact, there is considerable evidence to prove that environmental factors do influence the freezing point.

Buchanan and Lowman (19, 20) observed changes in freezing points with the seasons. They concluded that these changes were due primarily to changes in feed. However, they stated that meteorological conditions may have been a contributing factor. Regan and Richardson (78) observed that changes in environmental temperature affected the freezing point. Aschaffenburg and Veinoglou (9) suggested that differences in environmental temperature were a contributing factor to the seasonal variations that they observed.

Koenig (58) observed that cows on a semistarvation diet of poor-quality potato pulp gave milk with abnormally high freezing points. Rees (77) attributed most of the seasonal variations to differences in feed. Bagnall and Smith (11) observed that milk from cows which had recently been changed from stall feeding to grazing had abnormally high freezing points. Gaballah (39) also observed a change in the spring, with the shift to pasture feeding.

Pinkerton and Peters (1) observed that when a herd was shifted from a high- to a low-carbohydrate diet the freezing point of the milk was raised and the solids-not-fat was lowered.

Shipe, Dahlberg, and Herrington (86) observed differences in both the averages and ranges of freezing points of milk from five cows on three different rations. They observed similar results in another experiment involving nine cows on two rations. On the other hand, Kleyn *et al.* (56) did not find any significant difference between the average daily freezing points for milk produced on different rations. However, they did observe differences in the relationship between the freezing points of evening and morning milk for the different rations.

As was pointed out previously, Hortvet (45) assumed that the freezing point was independent of the breed of cows. However, there is evidence which raises questions about this assumption. Aschaffenburg and Veinoglou (9) noted that the freezing points of Guernsey milk were slightly higher than for Shorthorns. Krienke and Arrington (59) reported differences between Jerseys and Holsteins. Shipe, Dahlberg, and Herrington (86) reported differences between Brown Swiss and Holsteins. Bryant and Briggs (18) reported differences between Friesians and Guernseys. Peters, Mulay, and Shrode (70) reported differences between Holsteins and Jerseys. Sato *et al.* (81) determined the freezing point of milk

from five different breeds. They reported slight differences and pointed out that Ayrshire herds showed the highest average freezing point. Robertson (79) noted some differences between breeds in his national survey, but because of other variables it is difficult to evaluate the significance of differences in breeds. The amount of data on breed differences is insufficient to make any definite conclusions.

Notwithstanding Bailey's (12) observation that there were differences between the freezing point of morning and evening milk, it appears that some workers have assumed that there were no differences. This assumption was supported by the work of Stubbs and Elsdon (93), who reported an average freezing point of −0.545° C. for 440 morning samples and −0.544° C. for 550 evening samples. Kleyn and Shipe (55) found an average freezing point of −0.537° C. for 541 A.M. samples and −0.544° C. for 536 P.M. samples. Aschaffenburg and Temple (7) found that the average freezing point of morning milk was 0.002° C. higher than evening milk. In another study, Aschaffenburg and Veinoglou (9) found that the difference between morning and evening milk varied with the season. They found that in the winter the freezing point of evening milk was higher, whereas during the rest of the year the morning milk was higher. Shipe, Dahlberg, and Herrington (86) and Kleyn *et al.* (56) noted that the differences in freezing points between morning and evening samples varied with the ration. Kleyn also observed that the time interval between feeding and milking influenced these differences in freezing points.

Several workers (6, 43, 44, 49, 101) in England have shown extreme differences between morning and evening samples. These differences have been attributed to the fact that the cows had access to water only between the morning and evening milking. The morning milk appeared concentrated (i.e., had a low freezing point), whereas the evening milk appeared to be watered. The results of Aschaffenburg and Rowland (6) illustrate the type of differences obtained. On four different dates they found the freezing points of milk from a 32-cow herd to be −0.525, −0.528, −0.526, and −0.531° C. for evening milk; whereas, the corresponding values for morning samples were −0.555, −0.556, −0.560, and −0.566° C. This herd was kept in the barn from 4 P.M. to 9 A.M. and no water was available during this time. After the cows had been turned out to pasture, and had access to water both night and day, the freezing points were again determined on the mixed-herd milk. The values obtained on two different dates were −0.553 and −0.542° C. for the evening milk and −0.546 and −0.543° C. for morning milk.

It should be obvious from these findings on morning and evening differences that if one wishes to establish the authentic average for an individual cow or herd, it will be necessary to obtain samples of both morning and evening milkings.

A number of studies has been made to determine the effect of handling or treatment of milk on its freezing point. The effect of developed acidity was recognized by the early analysts. Bailey (13) studied this effect and noted an average freezing point lowering of about 0.003° C. for each 0.01% increase in acidity. Lythgoe (63) has suggested the use of a series of adjustment factors to correct for developed acidity. The official manual (10) states that "If the

titratable acidity, 15.4, exceeds 0.18%, results may underestimate the actual amount of added water in the sample." To prevent the development of acidity, Aschaffenburg, Temple, and Veinoglou (8) added 0.01% mercuric chloride. They applied a correction of 0.004° C. to those samples to which preservative had been added.

Rao, Dastur, and Dharmarajan (74) tested 11 preservatives and recommended the use of 0.075% mercuric chloride, for which they recommended a correction factor of 0.020° C. They reported an average difference of 0.019 ± 0.0082° C. between the original freezing point and the freezing point of milk, after the preservative had been added and the samples kept 1 wk.

While the development of acidity with storage has been recognized as a potential source of error, there is another, less-recognized effect of aging. Pinkerton and Peters (72) presented data showing the effect of storing ten samples of milk at 0° C. for 48 hr. In eight samples, the freezing point had changed and in all eight it had risen. The increases in freezing points ranged from −0.001 to 0.004° C. and were accompanied by decreases in conductivity. The author of this review has noted similar shifts in the freezing point when milk was stored for 24 to 48 hr. at 5° C. Where there were shifts, they were always up, indicating a decrease in the concentration of solutes. It is assumed that this is due, at least in part, to a decrease in the solubility of salts.

Freezing milk also has the effect of raising the freezing point. Shipe (84) reported results on two sets of samples which had been frozen and stored for 24 and 48 hr. He also noted that if one refreezes a milk sample, the freezing point of the second determination may be higher. A change in the freezing point was not observed in all cases, but in those where it did occur the freezing point of the replicate was always higher. On the basis of these observations, the author advises against refreezing an aliquot of milk as a duplicate.

A limited number of experiments have been conducted to determine the effect of heat on the freezing point. Monier-Williams (67) stated that heating for 20 min. at 60° C. raised the freezing point about 0.002° C. Elsdon and Stubbs (34) noted a slight tendency for the freezing point to be raised as a result of pasteurization or sterilization. However, the amount of change was small, and in some cases there was no change. Dharmarajan, Rao, and Dastur (28) reported that pasteurization did not affect the freezing point, but that boiling or simmering lowered it. This lowering may have been due to evaporation of water. Staub and Krahenbuhl (88) observed no change after pasteurizing with the holder method or after boiling for 10 min. under reflux. Pinkerton and Peters (72) reported that heating the milk to 161° F. for 16 sec. raised the freezing point and that heating to 169° F. for 16 sec. had a greater effect. They noted a corresponding decrease in the conductivity upon heating. Aschaffenburg *et al.* (3) reported that ultra-high-temperature treatment did not have any effect on the freezing point. Lazar and Henningson (61) observed that vacuum pasteurization raised the freezing point about 0.006° C. They attributed this shift to the removal of dissolved gases. Some of the changes noted by the other workers might be attributed to removal of dissolved gas. The lack of a detailed description of the

equipment used does not enable one to evaluate whether this could be an explanation for the apparent discrepancies. However, except for vacuum pasteurization, the changes observed by the various workers are so small that it is doubtful whether they have any practical significance.

The increasing use of vacuum treatment in processing milk does create a problem in interpreting freezing point data. However, except for the reference cited above there is no published information on this subject.

Doan (13) has shown that the freezing points of cream and skimmilk were the same as the original whole milk. However, they noted a relative sluggishness in the rate of rise of the mercury thread for the cream as compared with milk. Shipe, Roberts, and Blanton (87) have also observed differences in the apparent freezing rates for cream and milk. Their results suggest that the rate is related to viscosity. From a practical standpoint, the analyst should be sure to wait long enough after seeding viscous samples to insure that the maximum temperature has been reached.

Numerous papers have been published on the range and average freezing points. On the basis of the work and interpretations of Hortvet and Bailey (12, 45–48), the official average is −0.550° C. and the upper legal limit is −0.5335° C. in this country. The limitations of this work previously have been discussed. In a study involving 1,000 samples, Stubbs and Elsdon (93) in England obtained an average of −0.544° C., with a range from −0.529 to −0.563° C. Lampert (60) analyzed 20 samples of bulk milk and found an average value of −0.536° C., with a range of −0.529 to −0.540° C. On the basis of his findings, plus these of Hortvet, Bailey, and Stubbs and Elsdon, he recommended using −0.540° C. rather than −0.550° C. as the standard value.

Tankard and Bagnall (98), in a study of daily variations in the freezing point of evening milk over a four-day period, observed a range of −0.541 to −0.559° C. for 24 herd samples and −0.536 to −0.564° C. for 112 individual samples. Lythgoe (62) reported a range of −0.532 to −0.578° C., with a medium of −0.5535° C. for 73 authentic samples collected in Massachusetts during 1943-1944, and a range of −0.510 to −0.585° C. with a median of −0.5525° C. for 133 samples collected between 1920 and 1925. In a subsequent paper, Lythgoe (64) reported a range of −0.527 to −0.583° C. with a median of −0.548° C. for 148 individual cow samples collected between 1935 and 1946. Arrington and Krienke (2) reported a range of from −0.529 to −0.564° C. for 64 authentic samples.

Dharmarajan *et al.* (30), in India, reported an average freezing point of −0.548° C. for 535 herd and 307 individual samples. In another report on the composition of milk taken from different farms throughout India, Dastur *et al.* (27) reported an average of −0.545° C. for 202 samples.

Gaballah (39) collected 1,086 samples of individual and herd milks during 1950 through 1953 in Wisconsin. He reported an average of −0.546° C., with a range from −0.531 to −0.562° C. Sato *et al.* (81) reported an average of −0.546° C., with a range of from −0.533 to −0.560° C. for 59 authentic herd and individual cow samples collected in Ohio. Kleyn and Shipe (55) determined the freezing

point of 541 samples of evening milk from individual cows in the Cornell herd and reported an average of −0.541° C. with a range from −0.513 to −0.571° C.

Robertson (79) conducted a survey of the freezing point of milk from 16 areas in the United States and one in Canada. The reported average freezing point was −0.543° C. for 1,627 samples. The average freezing points reported from the separate areas ranged from −0.528 to −0.549° C. However, when the laboratories from the different areas determined the freezing point of two "standard milks," the differences between the reported values for the same samples of milk were of about the same order of magnitude as the differences between the "area milks." Therefore, it would seem that the analysts contributed as much to the variations in reported results as did the cows.

A number of workers have studied the freezing point of bulk milk which provides a basis for estimating the extent of adulteration. Aschaffenburg, Temple, and Veinoglou (8), in England, tested 4,000 samples of milk representing mostly individual producer samples. They stated that about six samples per hundred were found to contain water. From the distribution of freezing points, they concluded that it was unwise to calculate per cent of added water from an assumed mean of −0.550° C. They also noted definite seasonal effects—the freezing points were lower in May than during January to April.

Paley and Tzall (69), in New York City, determined the freezing points of 1,450 fluid milk samples obtained from milk dealers, their representatives, and duplicates of inspectors' samples. Only 40% of the samples showed freezing points within the 3% tolerance allowed by the A.O.A.C. Thirty-three per cent of the samples had freezing points between −0.535 and −0.530° C. On the basis of their data, they suggested that a study should be conducted to determine if the official value of −0.550° C. was valid for our present milk supply.

MacDonald (65), in England, collected 480 samples of milk from 3,000-gal. bulk lots over a period of a year and found that the freezing points varied from −0.542 to −0.548° C., with an average of −0.544° C. In a more extensive study between 1942 and 1947, he determined the freezing point of 9,543 samples of bulk milk and reported an average freezing point of −0.543° C. It should be noted that he did not report any freezing points below −0.550° C. in either of his studies.

Rees (76) reported a range of values from −0.514 to −0.570° C. for 155 samples of bulk market milk from one area of Tasmania, and a range of from −0.506 to −0.558° C. for 158 samples from another area.

Dahlberg, Adams, and Held (26) determined the freezing point of 135 samples of commercial pasteurized milk from eight cities located in different areas of the United States. These samples represented about eight million quarts of milk. The average freezing point of these samples was −0.540° C. The milk from two plants was definitely shown to be watered and excluding the results from these plants the average was found to be −0.541° C. Only 23 samples had freezing points of −0.550° C. or lower. They concluded that the distribution of freezing points cast some doubt on the A.O.A.C. standard of −0.550° C.

On the basis of lactometer readings and fat tests, Yongue (102) reported that 3,460 out of 28,368 samples of fluid milk were classified as suspected. The freezing points of 1,057 of these suspected samples were determined. Of these samples, 21.5% were found to have freezing points above −0.520° C. and 16.5% had freezing points between −0.520 and −0.529° C., inclusive.

Sato *et al.* (81) reported an average of −0.538° C., with a range from −0.450 to −0.550° C. for 108 samples of producers' milk, and an average of −0.530° C., with a range from −0.437 to −0.549° C. for 53 samples collected from retail outlets. They stated that 94% of the producers' samples would have been classified as unadulterated if −0.530° C. was used as the legal limit; whereas, only 87% would have been so classified if −0.534° C. was used. The retail samples showed 72 and 57%, respectively, by these two standards.

FREEZING POINTS OF THE MILK OF OTHER MAMMALS

There have been a limited number of studies on the freezing points of milk from other mammals. The most extensive studies have been made on buffalo milk. Dharmarajan *et al.* (30) reviewed the literature and reported that they had found an average of −0.552° C. for 193 individual and 271 herd samples of Indian buffalo milk. In another study of Indian buffalo milk, Dastur *et al.* (27) reported an average of −0.544° C. for 37 herd samples. Dharmarajan *et al.* (29) reported an average of −0.579° C. for goats' milk and −0.587° C. for ewes' milk. Kern (53), in Israel, reported an average of −0.565° C. for ewes' milk. Jax (51), in a study of the properties of sows' milk between the 2nd and 10th wk. of lactation, reported a range of freezing points from −0.528 to −0.683° C. Anselmi and Caló (1) obtained seven samples of elephants' milk during the 1st and 2nd wk. after parturition, and reported a range from −0.548 to −0.554° C. Miller and Ellis (66) determined the freezing point of 260 samples of milk from 215 healthy women and reported a range from −0.531 to −0.586° C., with a mean of −0.564 ° C.

FREEZING POINTS AS AN INDEX OF ADDED WATER

While the freezing points of the commercial samples provide an indication of the magnitude of the adulteration problem, the variations in the reported freezing points of authentic samples provide an indication of the problem of establishing appropriate standards.

In the past, Winter's table, as cited by Henderson and Meston (41), has been accepted by some as indicating the exact relationship between freezing points and percentage of added water. A literal interpretation of this table requires the assumptions that the freezing point of pure milk is constant and that the correct value is −0.550° C. Ample evidence has been cited to invalidate any assumption that the freezing point is constant. Furthermore, the evidence does not even support the assumption that −0.550° C. is the correct average. It should be pointed out that Winter's table was prepared before the development of the Hortvet method and that there is no published evidence to prove that Winter's and Hortvet's methods gave identical results.

One other criticism of Winter's table is that the calculations do not include any factor to compensate for the solids content of milk. His formula, which has been adopted by the A.O.A.C. (10), was:

$$\text{Per cent added water} = \frac{T-T'}{T}\,(100)$$

where T is the average freezing point (i.e., −0.550° C.) and T' is the observed freezing point. On the basis of their studies, Elsdon and Stubbs (36) have recommended the following formula:

$$\text{Per cent added water} = \frac{T-T'}{T}\,(100 - TS)$$

Bird and coworkers (15, 103) have shown the superiority of the Elsdon and Stubbs formula. To overcome the errors resulting from the use of too low an average, and from ignoring the solids content, Freeman (38) has recommended the following formula:

$$\text{Per cent added water} = \frac{(0.544 - T')\ 0.92}{0.544} \times 100$$

Regardless of the formula used, the problem lies in a choice of a value for the average freezing point. Obviously, the formula will be correct only if the proper value is chosen and in most cases this will not be known. Therefore, in most cases, the calculated percentage of added water represents only an approximation. To obtain a more reliable approximation, some regulatory agencies have adopted the practice of collecting authentic samples from the source of suspected samples.

Another means of verifying freezing-point results is to determine other properties of the suspected samples, such as the fat and solids content and the specific gravity. This procedure has been recommended by a number of workers (24, 62, 64, 79, 80). In some cases (24, 102), these tests are used for screening purposes, with the freezing point determination being used as a confirmatory test. Presumably, this practice has been followed because of the time consumed in running freezing points with the Hortvet apparatus. However, in view of the ease and speed of determining freezing points with the Fiske cryoscope, this test may be used more frequently for screening purposes. Since the freezing point test is a more accurate index of added water than other tests, it should be used as the test of first choice. In marginal cases, other tests, or comparison with authentic samples, should be made.

This brings us to the problem of establishing judicious freezing point limits. The British and Australians have been using −0.530° C. as an upper limit. The British Standards Institution (17) has recommended using an average of −0.540° C. for milk known to represent at least 200 gal., and an average of −0.530° C. for all other milks, in computing the percentage of added water. It recommends reporting this calculated value as "the minimum percentage of added water." This method of reporting calls attention to the fact that the calculated value is not necessarily the exact value. The use of different standards for large and small volumes of milk is at least partially justified, since abnormal

values from individual cows would be "diluted out" in bulk supplies. However, it is conceivable that a large herd could produce milk with an abnormal freezing point as a result of unusual environmental conditions. It is worth noting that the highest freezing point reported by Bailey (12) was the same (i.e., −0.530° C.) for both herd and individual cow samples.

Dahlberg (25) has suggested that −0.527° C. be fixed as the upper legal limit for the freezing point. [His choice of −0.527° C. was based on allowing 3% tolerance from the average of −0.543 ° C., obtained by Robertson (79) in his national survey.] Dahlberg points out that the laws regarding minimum standards for fat and solids serve as a precedent for this approach. Certainly, this approach would eliminate controversies about averages and the method of calculating added water. From the standpoint of fairness, it would be in the same category with the other minimum standards. In view of the normal variations, the fact that a sample meets minimum specifications does not necessarily indicate that the milk is free from added water. The British Standards Institution (17) also points this out in connection with its revised standard. The fact that a sample meets the minimum requirement would not prevent a regulatory agency from prosecuting if they had sufficient evidence of added water.

Robertson (79) has suggested the establishment of local standards as an alternative to a general standard. This alternative has merit if the freezing points vary consistently and significantly from one area to another. Of course, some of the apparent differences may be due to differences in analytical technique. This emphasizes the need to standardize the method. It would be unfortunate if a number of standards were established just to compensate for variations in the technique.

CONCLUSIONS

The observations of Hortvet and Bailey, that the freezing points of milk vary within relatively narrow limits, and that the freezing points of morning and evening milk may be different, have been substantiated.

Hortvet's conclusions that the average freezing point is −0.550° C. and that a 3% tolerance calculated from this value represents a reasonable standard, have not been substantiated. His assumption that different analysts will be able invariably to reproduce one another's results has not been verified. In fact, the literature indicates that a lack of agreement between analysts may be a significant cause of apparent variations in the freezing point of milk. His assumption that environmental factors do not affect the freezing point has been disproved.

The results indicate that the basic features of the Hortvet method are sound. However, the results indicate a need for more careful standardization of the method. The design of cryoscopes using thermistors as the thermal sensing elements may give impetus to the use of cryoscopic techniques, because of the ease and speed of making analyses.

In spite of the variations in freezing points and techniques, freezing points do provide an excellent index of added water. More general recognition of the limitations of the method, and judicious interpretation of the results, should help to avoid misinterpretations of cryoscopic results.

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CIRCULATION CLEANING. III. THE KINETICS OF A SIMPLE DETERGENT SYSTEM

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SUMMARY

Cleaning of milk films by solutions of sodium hydroxide is shown to follow the kinetics of a first-order reaction. A molar velocity constant is calculated, and the role of solution temperature is evaluated in terms of its effect on the rate of the reaction. The reaction between solutions containing hydroxide ion and milk films exhibits a Q_{10} of 1.6 under these conditions, and the effect of temperature on the reaction rate constant is linear, at least within the limits of 36 to 82° C. Application of kinetics to detergency permits temperature effects to be measured rather than estimated, and will allow studies of compatibility, incompatibility, or complementary interaction of detergent fractions, and selection of detergents best suited to particular requirements.

Although it is generally recognized that in most cleaning regimes the concentration of the detergent exercises an effect on the rate of soil removal, there has been no precise evaluation of this effect. This has been due, in part, to variability in soil films, the lack of a suitable method for accurately determining the amount of soil removed under standardized conditions, and the fact that such determinations do not yield precise points. Too, many of the preliminary investigations that have been made utilized plants in commercial operation. Studies made under these conditions have been handicapped, in that the cleaning regime under investigation must accomplish satisfactory cleaning; the most the investigator could hope to establish from such studies would be the minimal conditions to accomplish cleaning. It is not a simple matter to evaluate variables properly under this restriction.

Because the reaction(s) between films of deposited soil and detergent fractions would demand that the two species come in contact, the cleaning mechanism can be considered as somewhat analogous to a bimolecular reaction, and should be amenable to treatment from the standpoint of chemical kinetics. If this were true, not only could detergents be selected to do a particular cleaning job with greater efficiency, but the effect of variables such as temperature might be measured rather than estimated. Various authorities have recommended temperatures ranging from 115-180° F. for cleaning solutions (1, 2, 3, 6), and some (7) have pointed out that a major advantage of circulation cleaning is that higher temperatures are permissible. This work was directed to testing the application of chemical kinetics to detergency, and establishing methods for more precisely evaluating detergent efficiency. The studies reported herein were limited to the removal of milk films by hydroxide ion.

METHODS AND PROCEDURE

0.50-ml. aliquots of P_{32}-labeled milk were cooked on stainless steel test disc inserts machined to fit the inner periphery of a test section of 1½ o.d. stainless

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steel tubing (5). The amount of soil removed by particular cleaning regimes was followed by determining the decrease in the radioactivity of the test discs. Previous work has indicated that the radioactivity of films produced from in vitro labeled milk serves as a suitable index of both organic and inorganic milk residues (5).

The test apparatus used has been described elsewhere (4). It was modified slightly by coiling 10 ft. of ¾-in. o.d. copper tubing in the reservoir. This permitted temperature adjustment (by conducting cold water or steam through the coil) without dilution of the solution. The test section was on the pressure side (between the pump and the outlet) and possessed a positive slope of ¼ in/ft to insure flooded conditions. Solutions in 300-lb. lots were maintained at 46° C. (except as noted) and circulated at a Reynolds number of 550,000 (equivalent to 18.6 lb/sec or 29.4 ft/sec). These conditions of high turbulence minimized diffusion and concentration gradients of free hydroxide ion at the soiled surface, and assured a continuous supply of hydroxide ion at essentially constant concentration. The 300 lb. of solution contained 55, 165, 275, 550, and 825 g. AR NaOH for hydroxide ion concentrations of .01, .03, .05, .10, and .15 *M*, respectively. The .01 *M* solution was changed after every eight test discs, to insure an adequate reserve of NaOH. The water used possessed 120 p.p.m. hardness, as $CaCO_3$. Radioactivities of the soil films were determined with a thin-window Geiger tube in a Tracerlab SC 9 E shielded manual sample changer, coupled to an SC 1000 scaler.

RESULTS AND DISCUSSION

Hydroxide ion is undoubtedly involved in several reactions with these heterogeneous milk films but, kinetically, the over-all effect of hydroxide ion on cleaning should be closely related to a bimolecular reaction that can, after collision, proceed in different directions.

In the first analysis, hydroxide ion might be expected to limit its action to the exposed surface of the soil and, in effect, remove one layer of soil after another, exposing a fresh layer each time. If this were true, the rate of soil removal would be independent of the amount of soil until less than a monomolecular layer remained. Figure 1 indicates this is not the case, and that at a constant hydroxide ion concentration, the rate of soil removal decreases as the soil is removed. This could be interpreted as representing a saturation type phenomenon and, at least in the first stages, the reaction is probably first order in respect to soil. To avoid complications arising from a change in reaction rate with disappearance of the soil, determinations were, for the most part, limited to the disappearance of ca. 60% of the soil film.

It seems reasonable that under these test conditions, where the amount of soil is quite small in respect to the over-all amount of hydroxide ion available, and the latter is large enough that its concentration can be considered constant, the rate of soil removal also is probably first order in respect to hydroxide ion. Under these circumstances, the rate of soil removal could be expressed as

$$-\frac{dS}{dt} = k\,(S)\,(OH^-)$$

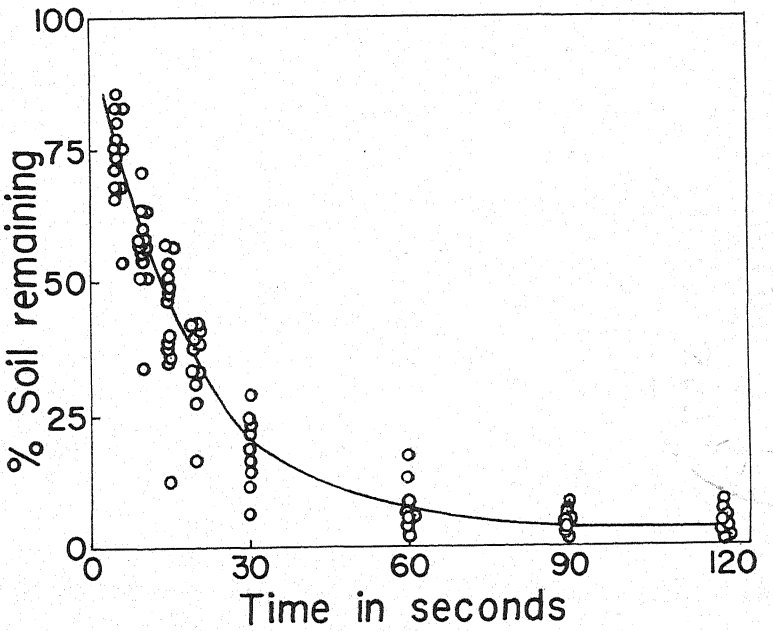


FIG. 1. Disappearance of soil with respect to time. (OH^-), 0.03 M; temperature, 46° C.; Re, 550,000.

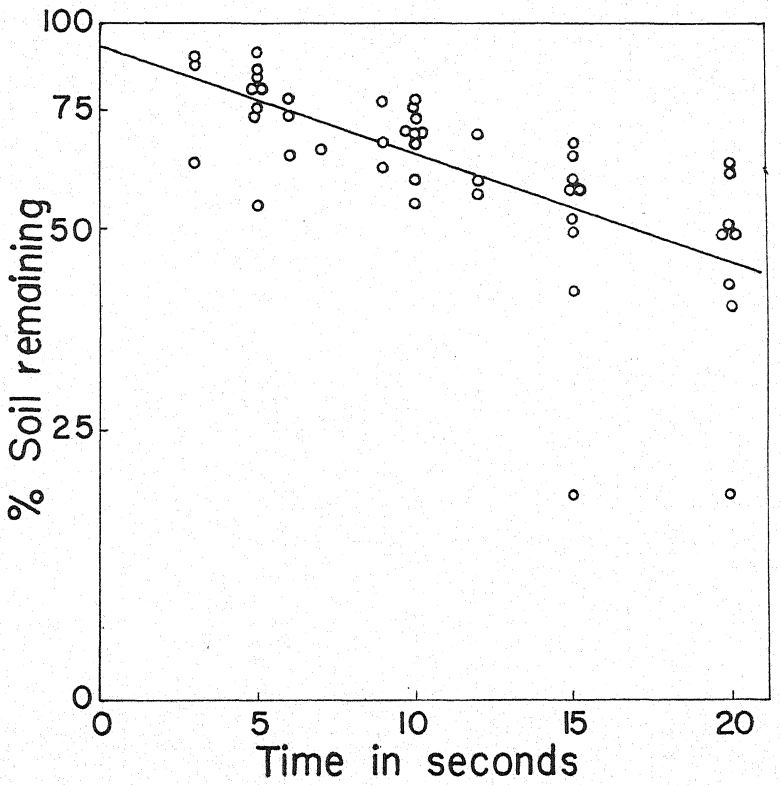


FIG. 2. Disappearance of soil with respect to time. (OH^-), 0.01 M; temperature, 46° C.; Re, 550,000.

where S denotes soil, expressed as per cent of the original deposit. There are theoretical objections to this approach, inasmuch as the unreacted soil is not in solution, but the results obtained and their high coefficients of correlation are ample justification. Ideally, the validity of this expression as regards hydroxide ion could be tested by constructing linear plots of S against t for various hydroxide ion concentrations. The tangents of these curves at some one value of S would equal $k\ (OH^-)$; this method would eliminate the effect of (S) on the expression.

However, determining the position of these curves is difficult because the data do not yield single precise points for a given value of t, but possess considerable scatter. By assuming first order kinetics and resorting to semilog plots, the best-fit lines can be calculated for the data. This approach was used in constructing Figures 2–6. The lines do not extrapolate through 100% soil because of a small inherent error in timing. The abscissae, time in seconds, represent pumping time under conditions of high turbulence. When the pump is shut off at the conclusion of a run, the fluid in the line drains under flow conditions that are essentially laminar. The correlation coefficients for the best-fit lines are listed in Table 1; each is highly significant. Included are the slopes of these best-fit

TABLE 1

Correlation coefficients and slopes for Figures 2–6

Figure	Hydroxide ion concentration	Slope	Correlation coefficient
2	.01 M	.016	.65
3	.03 M	.022	.89
4	.05 M	.027	.98
5	.10 M	.032	.87
6	.15 M	.043	.91

lines, as $-d \log S/dt$ at specific hydroxide ion concentrations. In Figure 7, the slopes are plotted as a function of hydroxide ion concentration. The line that fits these data best (method of least-squares) represents the activity of hydroxide ion in removing this type of milk film under these conditions.

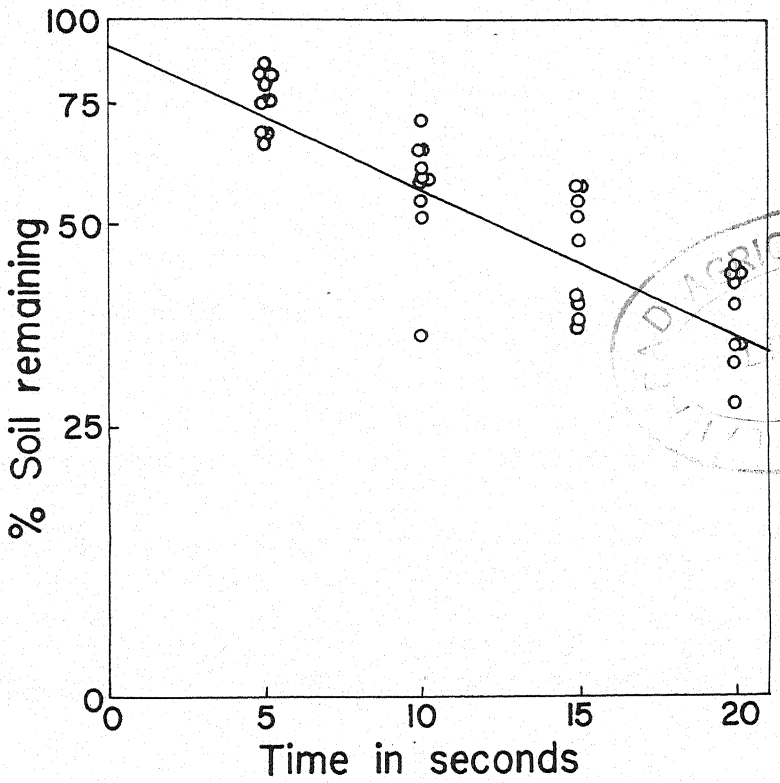
The equation for this line can be expressed as:

$$-\frac{d \log S}{dt} = k\ (OH^-) + C$$

where the constants have the values $k = 0.179$ sec.$^{-1}$ and $C = 0.016$ moles/liter/second.

The y intercept shows that soil removal is not entirely a function of hydroxide ion concentration. This is to be expected, for although we have an analogy to a bimolecular reaction, one species, S, will continue to disappear even in the absence of OH^-, due to the mechanical effects of solution turbulence, solution of soil constituents by the water phase, and other physical phenomena. The constant k might be termed the molar velocity constant of hydroxide ion on this type of soil.

Effect of temperature. Many attempts have been made to evaluate the effect of temperature on cleaning. Results in the literature (1, 2, 3, 5, 6, 7) are not



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FIG. 3. Disappearance of soil with respect to time. (OH^-), 0.03 M; temperature, 46° C.; Re, 550,000.

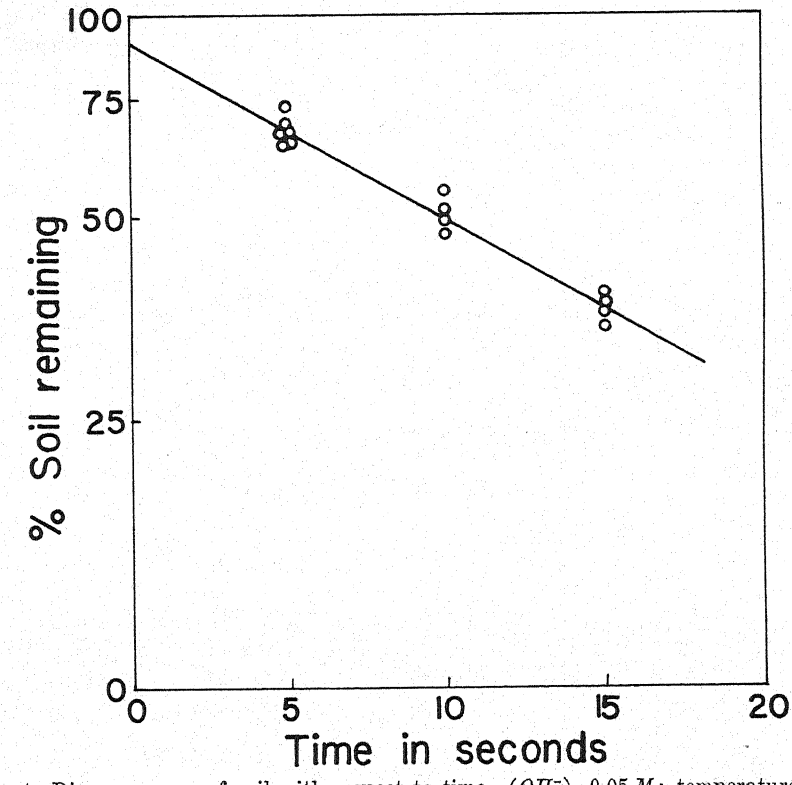


FIG. 4. Disappearance of soil with respect to time. (OH^-), 0.05 M; temperature, 46° C.; Re, 550,000.

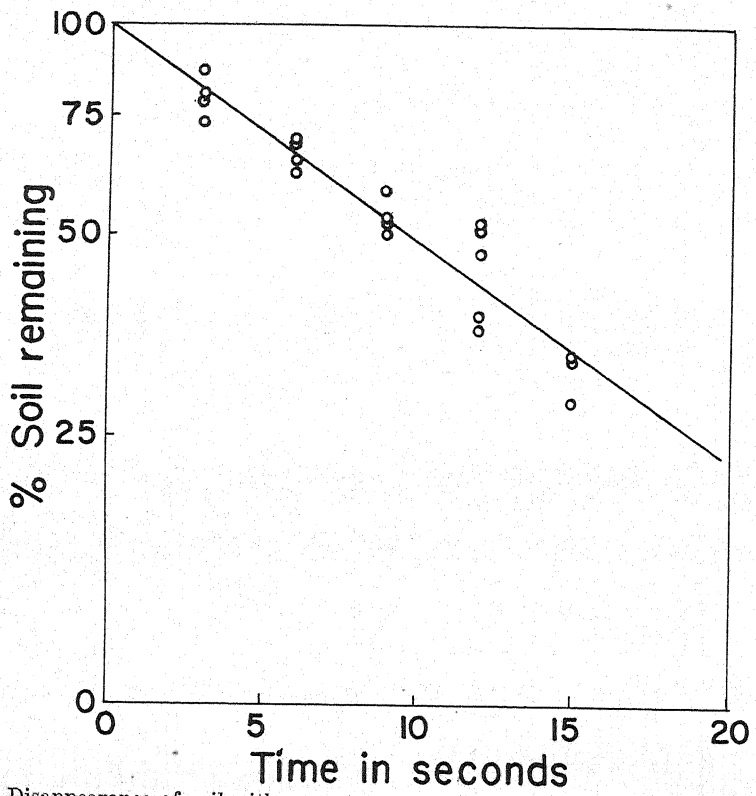


FIG. 5. Disappearance of soil with respect to time. (OH^-), 0.10 M; temperature 46° C.; Re, 550,000.

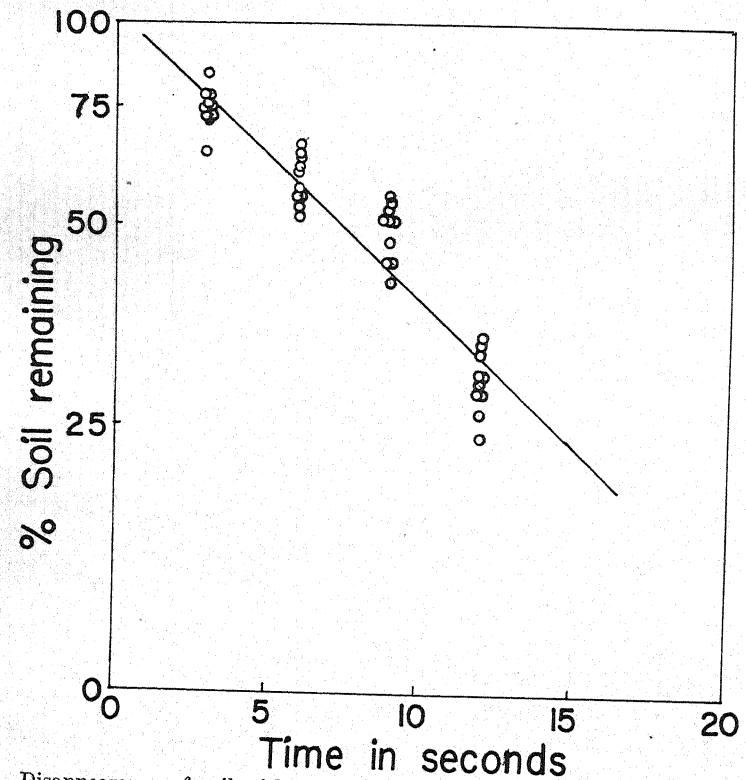


FIG. 6. Disappearance of soil with respect to time. (OH^-), 0.15 M; temperature, 46° C.; Re, 550,000.

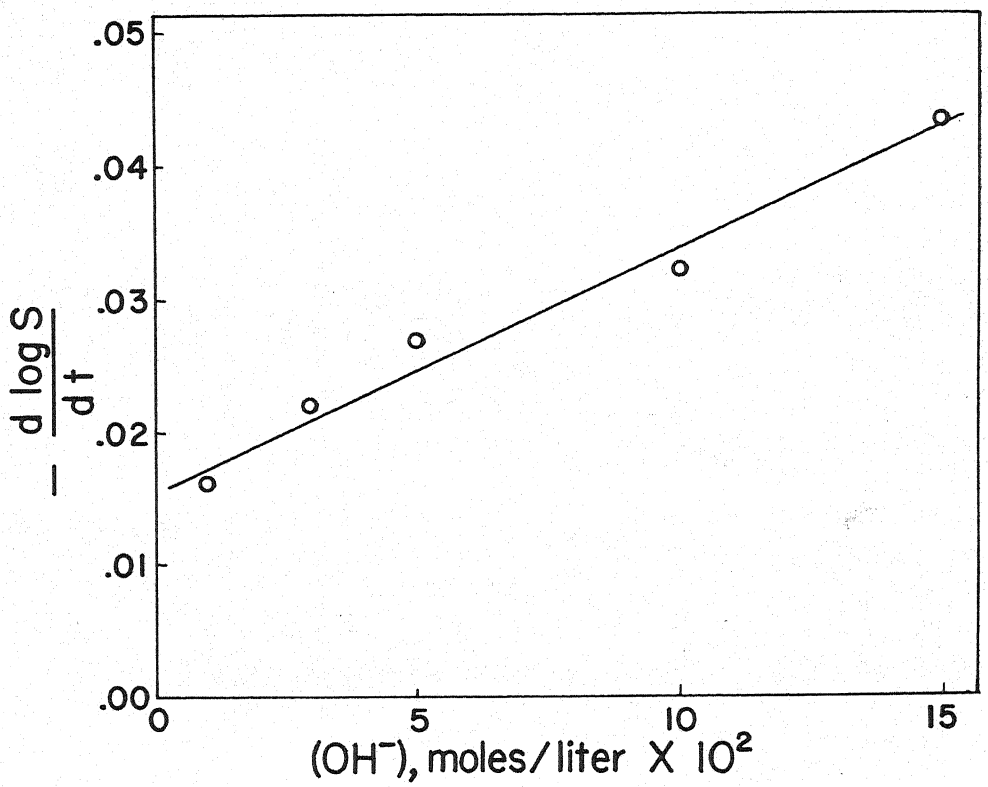


FIG. 7. Disappearance of soil with respect to time as a function of hydroxide ion concentration. Temperature, 46° C.; Re, 550,000.

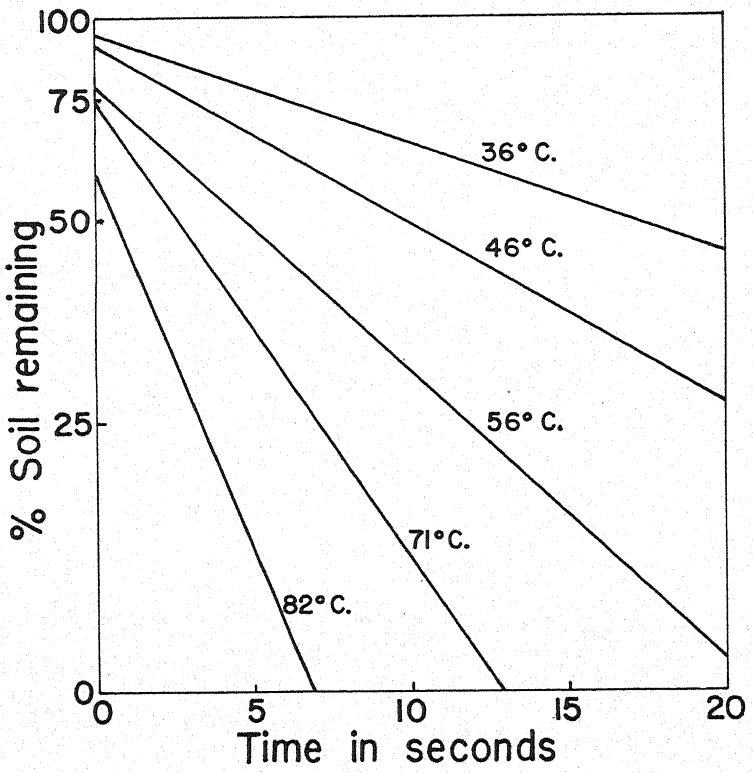


FIG. 8. Effect of temperature on rate of soil removal. Deterging medium, water; Re, 550,000.

in good agreement, largely because temperature effects could not be precisely evaluated. Series of determinations were made using .05 M sodium hydroxide at five different temperatures. All other experimental conditions remained the same.

Figure 8 illustrates the change in slope and y intercept as the temperature of the deterging medium is increased from 36 to 82° C. (these are best-fit lines, and individual points have been omitted for clarity), and in Figure 9(a) the the apparent linearity of this increase is demonstrated by plotting log k against the reciprocal of the absolute temperature. The change in slope (Figure 8) indicates that water is not inert, nor is its effect independent of temperature; at higher temperatures, it assumes a more important role.

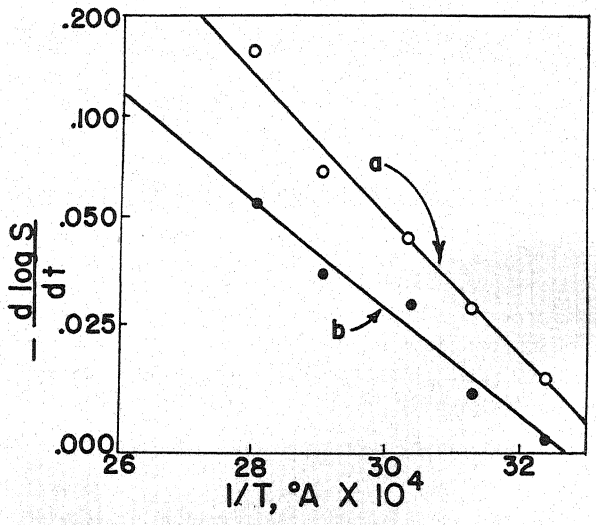


FIG. 9. Effect of temperature on rate of soil removal. Re, 550,000. a—.05 M (OH^-); b—water.

To properly evaluate the temperature variable, apparently it is necessary to set up an expression that takes into account the effect of temperature on (1) soil removed by the action of hydroxide ion, and (2) soil removed by action of the solvent, water:

$$-\frac{dS}{dt_{over\text{-}all}} = \frac{dS}{dt_{OH^-}} + \left(-\frac{dS}{dt_{HOH}}\right)$$

$-\frac{dS}{dt_{HOH}}$ can be evaluated for 46° C. by extrapolating Figure 7 to $(OH^-) = 0$, and at other temperatures by determining directly the rate of soil removal in the absence of hydroxide ion. Using techniques described above, series of determinations were made at several temperatures. Results are shown in Figure 9b.

From the formulas of the best-fit lines for these data [(a) $y = 7.898 - .0198\,x$; (b) $y = 6.557 - .0149\,x$)], it follows that

$$-\frac{d \log S}{dt_{over\text{-}all}} = 7.898 - \frac{.0198}{T_A}, \text{ and}$$

$$-\frac{d \log S}{dt_{HOH}} = 6.557 - \frac{.0149}{T_A}$$

Assuming that the cleaning effects of hydroxide ion and water are additive,

$$-\frac{d \log S}{dt_{OH^-}} = 1.341 - \frac{.005}{T_A}$$

these data point up the fact that in this range of concentration of hydroxide ion, water, far from being inert, is the most active detergent in the system (unless the comparison is on a mole-per-mole basis). While increasing temperature increases the effectiveness of both hydroxide ion and water, the increase is greater in the former. Solutions of hydroxide ion exhibit a Q_{10} of 1.6 on this type of soil.

CONCLUSIONS

It would appear that the phenomenon of cleaning can be treated from the standpoint of chemical kinetics, and that k values, or molar velocity constants, can be determined for the reaction of individual detergent fractions with specific types of soil. Determination of these constants permits a more precise evaluation of the effect of temperature on cleaning, the study of compatibility, incompatibility of complementary interaction of detergent fractions and, ultimately, should allow selection of a detergent or combination of detergents to best remove specific types of soil.

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ACTION OF PURIFIED MILK PHOSPHATASE ON PHOSPHOSERINE AND ON CASEIN

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SUMMARY

The action of a purified alkaline phosphatase from milk on casein and phosphoserine has been studied. The milk phosphatase preparation has no proteolytic action on casein, but it splits inorganic phosphate from both casein and phosphoserine. The action of the milk phosphatase was studied at pH 8 to 9.5 with several concentrations of phosphoserine. The optimum action was at pH 9.5 or higher. Values for the kinetic constants K_M and $V_{max.}$ were calculated from the data. The action of the milk phosphatase on phosphoserine was similar to its action on other simple phosphate esters. The milk phosphatase was considerably less reactive with casein (about 200 times more phosphatase was required for comparable release of phosphate), and the greatest activity was at pH 6 to 7. Reasons are given for not necessarily postulating a second enzyme for this activity, but rather that it might result from the complex nature and size of the casein molecule. Both calcium-sensitive α-casein and whole casein were dephosphorylated at comparable rates. The experiments were carried to 80% dephosphorylation of the casein, and presumably complete dephosphorylation could be accomplished with this phosphatase preparation. It is suggested that some dephosphorylation of casein might occur in milk and that special precautions may be required to obtain casein preparations of maximum and constant phosphorus content.

The availability of a purified, highly active milk phosphatase (23, 26) suggested that its action on casein be studied, since a number of other phosphatases (9, 11, 17) have been found to dephosphorylate casein. At the same time, the action of the milk phosphatase on serine phosphate has been studied, since most or all of the phosphate contained in casein is probably esterified to the OH group of this amino acid (8, 9, 18) or to the amino acid threonine (3).

MATERIAL AND METHODS

Whole casein. Whole casein was prepared by acidification of skimmilk to pH 4.5 with *N* HCl. The precipitate was washed four times with water, twice dissolved and reprecipitated with acid, then dried with absolute ethanol and ether (6).

α-Casein, calcium-sensitive. A calcium-sensitive α-casein was prepared from whole casein by a modification (24) of the fractionation procedure in aqueous urea solutions (7).

Preparation of alkaline phosphatase from milk. The alkaline phosphatase was prepared from skimmilk by the method previously described (23, 26). The preparation used in the present studies contained seven units of phosphatase activity per milligram of solids. A unit of phosphatase is defined as the amount which causes the liberation of 1 μM phenol in 5 min. at 38° C. The unit of activity

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defined here conforms to a recommended practice (10) and is about 300 times larger than the unit previously used (23). The phosphatase preparation contains about 2,500 units per milligram of the previously defined units (26). The dried phosphatase preparation was suspended in water to a concentration of 0.5% for the casein experiments, 0.1% for the phosphoserine experiments. The suspension was centrifuged and the supernatant solution, which contained all of the phosphatase activity, had a protein content (nitrogen × 6.25) equivalent to 70% of the dry weight of the phosphatase preparation, and the specific activity is about ten units per milligram of protein in solution.

Assay of alkaline phosphatase. The assays were run at 38° C. in an ethanolamine-HCl buffer of pH 9.8 with phenylphosphate as the substrate (25). The liberated phenol was determined colorimetrically (25) in the Beckman Model B spectrophotometer at a wave length of 650 mμ. The phenol equivalence was read from a standard curve relating phenol and color.

Action of the phosphatase on phosphoserine. The action of the phosphatase was determined by measuring the inorganic phosphate released from phosphoserine (commercial source) in 30 min. at 38° C. Two milliliters of the incubation mixture contained 1.6 ml. veronal-acetate buffer (12) in the pH range 8.0 to 9.5, 6 μM $MgCl_2$, 10 γ milk phosphatase, and 0.0125 to 5 μM phosphoserine. The reaction was stopped by the addition of 0.5 ml. of 0.38 N H_2SO_4. The inorganic phosphorus released was determined by the method of Dryer *et al.* (4). The optical density was measured in the Beckman DU spectrophotometer at 345 mμ and the phosphate estimated from a standard curve relating optical density and phosphate.

The action of phosphatase on caseins. The caseins, either whole casein or the calcium-sensitive α-casein, were dissolved using 0.1 N NaOH to obtain the approximate pH desired. Veronal-acetate buffer (12) of the desired pH was added and the pH was readjusted, if necessary, with 0.1 N NaOH or HCl. Five milliliters of incubation mixture contained 1.25 ml. buffer, 15 μM $MgCl_2$, 25 mg. casein, except where stated otherwise, and usually 5 mg. of the milk phosphatase. The solutions were incubated for various times at 38° C. The reaction was stopped by adding 1 ml. incubation mixture to 1 ml. 0.02 M silicotungstic acid, then 2 ml. of 20% trichloroacetic acid was added and the mixture centrifuged. The inorganic phosphate was determined on 2 ml. of the supernatant solution by the method of Sumner (21). The optical density of the developed color was measured in the Beckman DU spectrophotometer at 650 mμ.

RESULTS

The conditions for the optimum interaction of the alkaline phosphatase of milk with the substrate phosphoserine were investigated, with the thought that the results might suggest the best conditions for the interaction of alkaline phosphatase with casein. The phosphate of casein is probably bound through the hydroxyl groups of the amino acids serine or threonine, since phosphopeptides obtained from casein have the phosphate bound to the serine (8, 9, 18) or threo-

nine (3). The action of the phosphatase on phosphoserine was studied at several pH values, to determine the optimum pH, and also with several concentrations of the substrate, phosphoserine, to determine the enzyme-substrate dissociation constant, K_M, as a measure of the affinity between substrate and enzyme. The latter also provided data for estimating the concentration of substrate, giving the maximum velocity, $V_{max.}$, for the reaction. At a concentration of phosphoserine suitable for assay purposes, namely 125×10^{-5} M per liter, the pH optimum had not been reached even at pH 9.6. As with other alkaline phosphatase reactions, the pH optimum is a function of substrate concentration and with 25×10^{-5} M phosphoserine per liter, the optimum is at pH 9.0.

The K_M and $V_{max.}$ for the action of the milk phosphatase on phosphoserine were computed from the integrated form of the Michaelis-Menten equation developed by Walker and Schmidt (22).

$$(S_o - S_t) \cdot t = -K_M (2.3 \log S_o/S_t) \cdot t + V_{max.}$$

S_o is the initial substrate concentration, and S_t is the substrate concentration after the action of the enzyme for time t. By a plot of $(S_o - S_t) \cdot t$ versus $(2.3 \log S_o/S_t) \cdot t$, a straight line is obtained. The slope is $-K_M$ and the intercept on the $(S_o - S_t) \cdot t$ axis is $V_{max.}$ The use of this equation made it possible to include data at low substrate concentrations for the calculation of K_M and $V_{max.}$, a substrate region where reaction rates are linear for only a brief time. The results are presented in Figure 1, where pK_M $(= 1/\log K_M)$ is plotted against pH, and in Figure 2, where $V_{max.}$ is plotted against pH. The large pK_M value at pH 8 means that the substrate concentration has to be less than 2.5×10^{-5} M

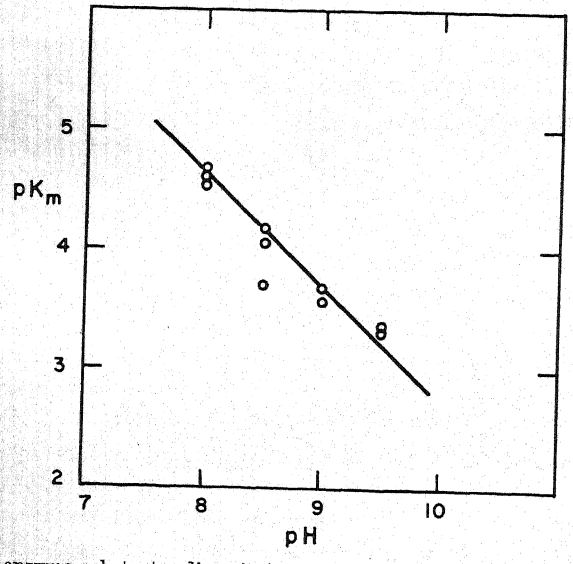
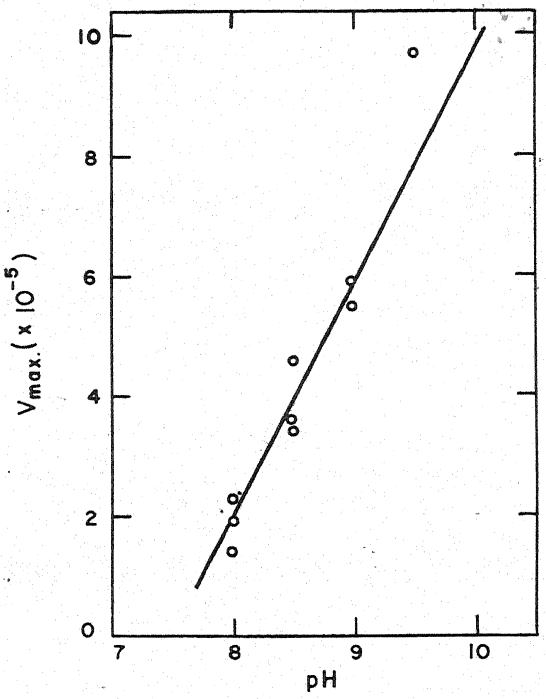


FIG. 1. The enzyme-substrate dissociation constant $(pK_M = \log 1/K_M)$ for the action of the milk alkaline phosphatase on the substrate phosphoserine at various pH values. K_M is expressed in moles per liter.



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FIG. 2. The maximum velocity, $V_{max.}$, for the action of the milk phosphatase on phosphoserine at various pH values. $V_{max.}$ is expressed in moles per liter per 5 min.

per liter before the reaction becomes dependent on substrate concentration. This made it impractical to estimate the K_M values at pH values below eight, since these concentrations of inorganic phosphate released were too small to be estimated accurately.

The results with phosphoserine, although of general interest, did not serve as a guide for the action of the milk phosphatase on casein. The milk phosphatase preparation did dephosphorylate casein, but much larger concentrations (about 200 times) of phosphatase were required than for phosphoserine, and dephosphorylation of casein was more rapid at pH 6 than at pH 10. A detailed comparison of the action of the milk phosphatase on the two substrates will be discussed later.

Both whole casein and calcium-sensitive α-casein were dephosphorylated to about the same extent. The results with the two caseins and several concentrations of the milk phosphatase are shown in Figure 3. The reason for the nonlinear release of phosphorus as the amount of phosphatase is increased is not clear. It was not due to a decrease in the phosphatase activity, for all of the activity (tested with the substrate phenylphosphate) was still present at the end of the experiment. In fact, the casein stabilized the phosphatase, for phosphatase stored at this pH and temperature for the same length of time with no casein present had lost some activity. The ninhydrin reaction (13) applied to this

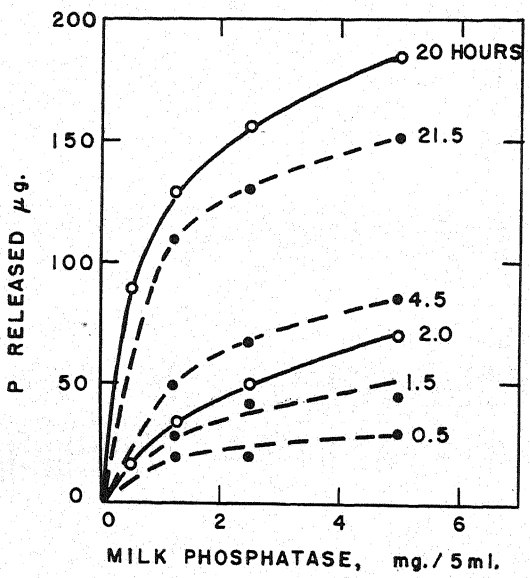


FIG. 3. Action of milk prosphatase on whole casein (pH 7.0) and calcium-sensitive α-casein (pH 6.4) for various periods of time, measured by the release of inorganic phosphate. ●——● Whole casein (177 γ casein-P in 5 ml.); ○——○ α-casein, calcium-sensitive (250 γ casein-P in 5 ml.). Numbers at ends of curves indicate hours that the phosphatase acted.

mixture remained the same after 0, 2, and 20 hr.; hence, the milk phosphatase preparation contained no proteolytic activity for the conditions used.

The data in Figure 4 show the influence of time and pH on the dephosphorylation of calcium-sensitive α-casein by the milk phosphatase. The maximum rate of hydrolysis results at pH 6 and 7. The shape of these curves—rapid initial hydrolysis followed by a considerably slower rate—is characteristic of this dephosphorylation.

The effect of the concentration of casein on the dephosphorylation activity of the milk phosphatase is shown in Figure 5 for pH 6.5 and 9.0. K_M and $V_{max.}$ values were calculated by the procedure described earlier for phosphoserine. Straight lines were obtained when $(S_o - S_t) \cdot t$ was plotted against (2.3 log $S_o/S_t \cdot t$; hence, for these conditions, the catalytic activity of the phosphatase appears to represent the usual summation of zero- and first-order rates.

DISCUSSION

Chemical properties of phosphoserine have been described (16) and a few reports of the action of phosphatases on this compound have appeared. A preparation from calf intestinal mucosa will dephosphorylate phosphoserine as rapidly as it does glycerophosphate (19), presumably due to the alkaline phosphatase which is present in intestinal mucosa in large amounts. Phosphatases also have been reported (15, 20) that are specific for phosphoserine, acting only on this substrate. They will also phosphorylate serine.

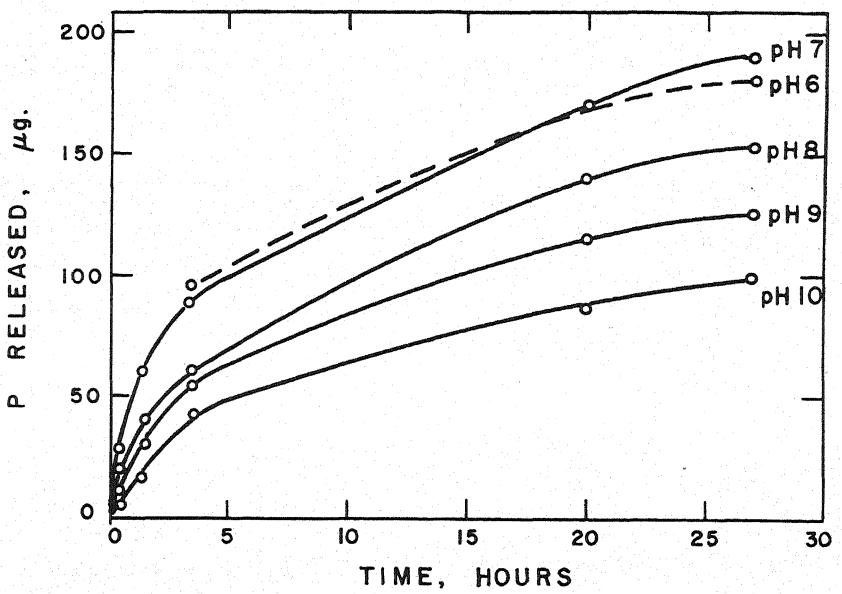


FIG. 4. Action of milk phosphatase on calcium-sensitive α-casein at several pH values, for various periods of time, measured by the release of inorganic phosphorus. The amount of milk phosphatase in this experiment was 2.5 mg. in the 5-ml. reaction mixture.

The present studies show that the action of the milk alkaline phosphatase on phosphoserine is very similar to its action on other simple phosphate esters. Phosphoserine and phenylphosphate are hydrolyzed equally rapidly by the milk phosphatase (23); with phosphoserine, however, the phosphatase has the same

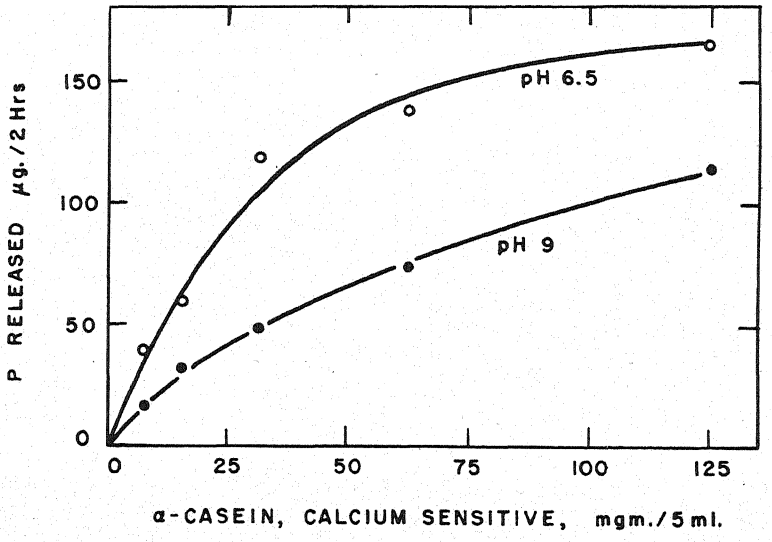


FIG. 5. Action of milk phosphatase on calcium-sensitive α-casein with several concentrations of the casein at pH 6.5 and 9.0, measured by the release of inorganic phosphorus in 2 hr.

pK_M-pH values as with *o*-carboxyphenylphosphate (23). The milk phosphatase, acting on phosphoserine, also has its greatest activity at pH 9.5 or above. The milk phosphatase preparation used in the present study contained no acid phosphatase, that is, a phosphatase active at pH 4, which has been reported in milk (14). Large amounts of the milk phosphatase were incubated at 35° C. at pH 4.0 with phenylphosphate and with phosphoserine with negative results.

The information about the action of the milk phosphatase on phosphoserine provided no guide to its action on casein. The milk phosphatase does dephosphorylate casein, but large amounts of the phosphatase are required, and the most marked difference is that dephosphorylation is more rapid at pH 6 to 7 than at pH 9.5. As noted above, the phosphatase preparations did not contain an acid phosphatase, but the above pH data suggest that a second dephosphorylating enzyme might be present. Previous studies (26) had showed that the purified milk phosphatase preparations contained an enzyme-hydrolyzing nucleic acid, and there well might be other phosphorylitic enzymes in the milk phosphatase preparations also. The pH data, however, do not necessarily require that the enzyme-dephosphorylating casein be different from the enzyme-hydrolyzing phosphoserine and other simple phosphate substrates. The casein is a large molecule and there might be a local charge on its molecule at the higher pH values that would hamper enzyme-substrate union and consequent dephosphorylation of the casein.

At pH 6, on the other hand, the unfavorable charge might be absent, thus permitting action of the phosphatase, although at a low level. A phosphatase preparation from calf intestinal mucosa will dephosphorylate casein (11) but here, too, although it is a potent source of alkaline phosphatase, the greatest activity on casein is at pH 7.4. A kinetic analysis of the action of the milk phosphatase on casein (Figure 5) shows that this action has a greater affinity between enzyme and substrate (smaller K_M value) at pH 6.5 than at pH 9.0. In this respect, the effect of pH is similar to the effect on the action of the phosphatase on phosphoserine. The actual values for K_M are 1.5 mM casein-P per liter (0.5% casein) at pH 6.5, and 4.5 (1.5%) at pH 9.0. These values are of the same magnitude as others have reported for enzymatic dephosphorylation of casein. The value for the spleen phosphatase acting on casein at pH 5.5 was 3 mM casein-P per liter (9).

Calcium-sensitive α-casein was chosen for the present studies to have a casein that was free of other interacting components. This choice was motivated by the report (17) that whole casein was very poorly dephosphorylated by phosphatase, whereas the separated α- and β-caseins were readily dephosphorylated. Recent studies (9, 11), however, have not confirmed this claim, and the present studies show that the calcium-sensitive α-casein and whole casein were dephosphorylated at comparable rates.

The results of the present study raise the question whether the action of the phosphatase in milk might lead to caseins of variable phosphate content, depending on the time of isolation after milking and other factors. The amount of phosphatase used in the present studies, although large, to obtain a readily measur-

able dephosphorylation, is only about ten times greater than the concentration found in milk. There is about 0.7 unit (defined above) of phosphatase per milliliter of milk (0.7 unit to about 30 mg. casein); whereas, the purified phosphatase contained seven units per milligram and, as shown in Figure 3, 1 mg. of this phosphatase gives a marked dephosphorylation of 25 mg. of casein.

It is well known that the inorganic phosphate in milk increases on standing, but analysis indicates that all of this increase seems to be accounted for by a decrease in the soluble, low molecular weight phosphate esters in milk (5). The relatively large contribution of this fraction to an increase in the inorganic phosphate might, however, mask a contribution from dephosphorylation of casein. Changes in the phosphatase content of cow's milk by the administration of thyroxine or thiouracil bring about changes in the phosphorus content of the casein (1). Thyroxine decreases the phosphatase in milk and the phosphate content of the casein goes up; thiouracil increases the phosphatase content of milk (as much as threefold above normal) and the phosphate content of casein goes down. A somewhat similar pattern emerged from changes in the phosphatase during the course of lactation (2). The authors (1) attributed the changes in the phosphate content of casein to metabolic changes within the mammary gland. The present studies, however, suggest that the change in the phosphate content of casein may have taken place after the milk was formed. Also, it appears that special precautions will be required to obtain casein preparations of maximum and constant phosphate content.

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PSYCHROPHILES IN MILK HELD TWO DAYS IN FARM BULK COOLING TANKS [1]

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SUMMARY

Predominant psychrophilic bacteria were isolated from 174 samples of every-other-day milk from bulk milk cooling tanks from 12 farms producing Grade A milk. The 220 isolates so obtained were identified as to genus and species as far as possible. For the most part they fell into the genera *Aerobacter, Alcaligenes, Arthrobacter, Flavobacterium, Micrococcus,* and *Pseudomonas.* Species of these genera are listed. Not all of the species could grow in pasteurized milk within three days at the cooling tank temperature of 38° F. (3.3° C.), although they formed colonies in seven days at 5° C. on plate count agar, and most of them could grow in pasteurized milk at 45° F. (7.2° C.) All of the species grew little, if any, in raw milk in the cooling tanks at 38° F.

During barn feeding and holding of the cows, *Arthrobacter* species usually were predominant in the every-other-day milks, with *Pseudomonas* second in frequency and *Micrococcus* third. While the cows were on pasture, bacteria of the genus *Flavobacterium* were most often predominant, followed by *Arthrobacter,* and *Alcaligenes* first was detected. There was an average sevenfold increase in total numbers of psychrophiles when the cows went on to pasture from barn housing. There seemed to be a tendency for a certain genus of psychrophile to predominate in milk from a given farm, although the method of holding and feeding, barn versus pasture, had an important influence.

Marth and Frazier (2) reported that the predominant psychrophiles in most high-count, raw market milk handled chiefly in pipeline milkers were in the genera *Pseudomonas, Alcaligenes, Achromobacter,* and *Flavobacterium.* By contrast, the milks in the present studies were low-count, for the most part, and did not contact farm pipelines. The genus *Micrococcus* was not reported by Marth and Frazier, but often is listed among the genera containing psychrophiles.

METHODS

Incidental to the estimation of psychrophilic plate counts on every-other-day milk from the bulk cooling tanks of 12 farms from the Chicago milkshed (1), plates became available for the isolation of psychrophilic bacteria. Psychrophiles in this instance were considered to be bacteria that formed visible colonies in plate count agar (*Standard Methods for the Examination of Dairy Products,* 10th ed. 1953) after incubation for seven days at 5° C. The milk from the 12 farms was produced to meet Grade A standards and, for the most part, had low standard plate counts and psychrophilic plate counts. Arithmetic means of the standard plate counts of all every-other-day samples of patrons' milks ranged from 4,700 to 110,000 per milliliter and had an over-all average of about 27,000 per milliliter. Arithmetic means of the psychrophilic plate counts ranged from 350 to 4,800 per milliliter and averaged 2,800 per milliliter.

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[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] At present with The Quaker Oats Co., Barrington, Ill.

All farm bulk cooling tanks were refrigerated, so as to maintain a milk-storage temperature of approximately 38° F. (3.3° C.), except briefly at milking time. Two-day-old milk samples were collected at the 12 farms and iced during transportation to the laboratory, where they were plated for the psychrophiles. From each sample of milk a single plate culture of the highest dilution showing appreciable growth was chosen and examined for predominant colonies. One colony of each predominant kind was picked and purified, and an attempt was made to identify the bacterium. Altogether, 220 isolates were obtained from 174 samples of every-other-day milk from the 12 farms. The bacteria were classified into genera and, if possible, into species, from descriptions outlined in Bergey's *Manual of Determinative Bacteriology,* 7th ed. 1957. Nine isolates were not identified as to genus.

RESULTS

It will be observed in Table 1 that *Arthrobacter* was predominant most often, making up about 37% of the isolates, and *Flavobacterium* next most often, comprising about 29% of the isolates. Next came about 17% of *Pseudomonas,* followed by still fewer bacteria of the genera *Micrococcus* and *Aerobacter.* Only twice was *Alcaligenes* predominant. The species tested under the various genera fitted the descriptions in Bergey's *Manual,* except for an occasional minor characteristic.

A test was made of the ability of representatives of some of the above genera to grow within three days at 38° F. (3.3° C.) in pasteurized milk. None of the species of *Arthrobacter, Micrococcus,* or *Flavobacterium* tested was able to grow. Fairly rapid growth of *Alcaligenes marshallii, Pseudomonas segnis, Pseudomonas convexa,* and *Pseudomonas cohaerens* was observed, and almost as good growth of *Aerobacter aerogenes. Pseudomonas schuylkilliensis* showed a two-day lag before

TABLE 1

Frequency of isolation of genera and species of psychrophiles from every-other-day milk

Genus	Species	Total No. of isolates	Percentage of total isolates
Arthrobacter		81	36.8
	A. simplex	59	26.8
	A. citreus	11	5.0
	Unidentified	11	5.0
Flavobacterium		63	28.6
	F. aquatile	61	27.7
	F. suaveolens	2	0.9
Pseudomonas		37	16.8
	P. schuylkilliensis	8	3.6
	P. convexa	7	3.2
	P. cohaerens	17	7.7
	P. putrefaciens	2	0.9
	P. segnis	3	1.4
Micrococcus		16	7.4
	M. candidus	9	4.1
	M. caseolyticus	3	1.4
	M. conglomeratus	4	1.8
Aerobacter		13	5.9
	A. aerogenes	9	4.1
	A. cloacae	4	1.8
Alcaligenes	*A. marshallii*	2	0.9
Unidentified		9	4.1

initiating growth, and *Pseudomonas aquatile* failed to grow. Most of the species unable to grow within three days at 38° F. (3.3 C.) could grow in pasteurized milk at 45° F. (7.2° C.). It should be reported, however, that even those species able to grow well at 38° F. in pasteurized milk apparently grow little, if at all, in the raw milk in the cooling tanks, for psychrophilic plate counts averaged as low for one-day milks as for every-other-day milks.

It was observed that a certain genus of psychrophile tended to predominate in milk from a given farm, although, as will be shown, the method of feeding, barn versus pasture, had an important influence. Milk from seven farms had an over-all predominance of bacteria of *Arthrobacter* species, although they were predominant less often during pasture feeding than during barn feeding. Milk from three farms had *Flavobacterium* species predominant most often; one farm had mostly *Pseudomonas* and one chiefly *Aerobacter*.

During the period of the experiments (March 3 to June 8), the cows were changed from barn feeding to pasture feeding. Table 2 shows the predominant genera found in the every-other-day milk samples during the two periods. During the time that the cows were fed in barns, more isolations were made of bacteria of the genus *Arthrobacter* than of any other genus, with *Pseudomonas* second in frequency and *Micrococcus* third. On the other hand, after the cows went on to pasture, bacteria of the genus *Flavobacterium* were predominant, followed by *Arthrobacter;* and *Alcaligenes* first was detected in two samples. Total average numbers of psychrophiles increased from 920 per milliliter during barn feeding to 6,700 per milliliter during pasture feeding.

TABLE 2

Comparison of predominant genera of psychrophiles in every-other-day milk from 12 patrons during barn feeding and pasture feeding

Psychrophiles	Percentage of total cultures isolated	
	Barn feeding[a]	Pasture feeding[b]
Arthrobacter spp.	51	24
Flavobacterium spp.	6	56
Micrococcus spp.	10	5
Pseudomonas spp.	25	9
Aerobacter spp.	8	4
Alcaligenes spp.	0	2

[a] Eight-week period.
[b] Following 4-wk. period.

DISCUSSION

Apparently there was little, if any, growth of psychrophiles during holding in the cooling tank in most of the milk samples tested. Therefore, the higher numbers present in the milk during pasture feeding must have been caused primarily by contamination additional to that received in the barn at milking while the cows were being held in the barn. Present results indicate that this additional contamination consisted chiefly of yellow bacteria that have been classified here as *Flavobacterium* species. According to Bergey's *Manual,* these bacteria come mainly from water or soil, and *Arthrobacter* species from soil. It

has been known for some time, however, that the surface flora of green plants contains a considerable proportion of yellow-pigmented, gram-negative, nonsporeforming rods that Wöller (3) and others have called *Bacterium herbicola* and the sixth edition of Bergey's *Manual* calls *Pseudomonas trifolii*. However, the yellow, gram-negative bacteria encountered in the present work and classified as *Flavobacterium*, were nonmotile, according to tests; whereas, all *Pseudomonas* species tested had polar flagella. It may be that *B. herbicola* is really a *Flavobacterium*, and that green plants are one source of these bacteria.

Micrococcus and *Arthrobacter* were found in the present study and not in that of Marth and Frazier (2). For the most part, the predominating bacteria of the every-other-day milk in the present experiments were those which predominated in the original contamination of the milk, for little, if any, growth took place. Bacteria of the two above-mentioned genera were unable to grow in the milk held at 38° F., but grew on the plates of the psychrophilic plate count. On the other hand, Marth and Frazier worked with milk most samples of which were heavily contaminated at the start, with the farm pipelines as an important source of bacteria. Also, there was growth of psychrophiles in a number of samples.

ACKNOWLEDGMENTS

Thanks are due J. C. Werren, whose plates were used for isolation of psychrophiles, and to the Associated Milk Dealers, Inc., of Chicago, Illinois, for partial support of the project.

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RELATION BETWEEN LACTIC ACID AND DIRECT MICROSCOPIC COUNTS FOR BACTERIA IN NONFAT DRY MILK[1]

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SUMMARY

About 50 dry milk samples with known direct microscopic counts for bacteria were analyzed for lactic acid by Ion exchange, Golden State, Davidson, and Ling methods. Precision was poor on all but the Ion exchange method. All methods gave correlation coefficients of about .8 between apparent lactic acid and bacteria counts. The Ion exchange method was found to be the most accurate and also the slowest. The Ling method was definitely the fastest. The value of lactic acid content as an estimate of bacteria count was calculated. The expected range of bacteria counts for a given amount of lactic acid was too large for lactic acid to be a satisfactory indicator of bacterial contamination. Roller-dried samples gave unexpectedly high analyses for lactic acid.

The most obvious chemical change brought about by bacteria in milk is usually the production of lactic acid. There has been a persistent interest over the years in the relationship between bacteria counts and lactic acid production.

Hammer and Hix (4) found only a very general relationship between the rise in acidity and the numbers of organisms present in sterile milk inoculated with lactic acid bacteria. Similar results were found by Gould and Jensen (3) in random samples of milk which were taken as received at a creamery and allowed to stand at 22–24° C. until noticeable off-flavors developed.

In the present study, we have used four recently developed methods for lactic acid determination to analyze samples of nonfat dry milk of known direct microscopic counts for apparent lactic acid. About 50 spray-dried samples were analyzed in duplicate by the Golden State (7), Davidson (2), and Ling (5) procedures. Also, single analyses were run on 25 samples by the Ion exchange method (9). The reproducibility of each of the four methods was determined by statistical analyses of the data and an estimate was made of their accuracy. Correlation coefficients between the direct microscopic clump counts and the apparent lactic acid present also were calculated for each method. The approximate time required to run a set of samples of each method was recorded and the probable usefulness of lactic acid content in estimating the direct microscopic count was evaluated statistically.

MATERIALS AND METHODS

In Davidson's method (2), the reconstituted milk is deproteinized and desugared by treatment with copper sulfate and lime. The filtrate is treated with sulfuric acid to convert lactic acid to acetaldehyde. Color is developed with copper sulfate and *p*-phenylphenol and measured in a spectrophotometer.

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[1] Presented in part before the American Dry Milk Institute Research Conference in Madison, Wisconsin, on October 13, 1958.

In the Golden State method (7), the sample is first deproteinized with tungstic acid and then carried through the Davidson procedure.

The Ion exchange method (9) involves first extracting the lactic acid from the dry milk powder with 80% methanol. The extract is then passed through the cation and anion exchange columns. The eluate from the anion column is treated with sulfuric acid and *p*-phenylphenol to develop the color which is measured in a spectrophotometer.

The above procedures were carried out essentially as described in the original references, which may be referred to for details of the procedures.

A modified Ling method (5), based on a procedure developed by the American Dry Milk Institute (1), was used in our study. A more satisfactory modification of the Ling method since has been developed by the A.D.M.I. (1).

In the procedure used, 5.38 g. of dry milk was reconstituted in 50 ml. of water and a 25-ml. aliquot taken. This was deproteinized by adding successively 10 ml. of barium chloride solution (98.8 g/liter), 5 ml. zinc sulfate solution (225 g/liter), and 10 ml. 0.66 *N* sodium hydroxide, and filtering. One milliliter of 1% ferric chloride in 0.025 *N* hydrochloric acid was added to 10 ml. of the filtrate. The color was read after 30 min. with optical densities taken at 425 and 650 mμ. The difference was used to determine the lactic acid present. The reading at 650 mμ was to correct for turbidity which frequently developed in the final solution.

The calibration curve was prepared by adding known amounts of calcium lactate to a milk sample and measuring the increase in optical density.

A filtrate prepared by the Davidson procedure was chromatographed to determine whether color-producing substances other than lactic acid were present. For this experiment, 1 g. of sample was deproteinized by the Davidson method and the solution volume made up to 100 ml. The solution was filtered and the pH of the filtrate (66 ml.) was adjusted to 4.5 and passed through the cation column, and the column washed with water. The pH of the cation effluent and combined wash water (approximately 200 ml.) was adjusted to pH 8 and passed through the anion column and the column washed. The volume of anion effluent and combined wash water was adjusted to 300 ml. and labeled nonionic fraction. The cation column was then eluted with 200 ml. *N* HCl and the anion column eluted with 200 ml. *N* Na_2CO_3. The color determination in the various fractions was then made as described in the Ion exchange method.

All colorimetric readings were made with a photoelectric colorimeter with matched 6-in. test tubes.

RESULTS AND DISCUSSION

The statistical data for the various methods are summarized in Table 1. Of the four methods used, the Ion exchange method (9) is the only one capable of good reproducibility. The Ion exchange method gives distinctly lower results than the three other methods. It effectively separates lactic acid from interfering materials and is probably the only method that measures true lactic acid accurately. However, it is too slow for routine work.

TABLE 1
Statistical data for lactic acid analyses by four methods

	Ion exchange	Ling	Davidson	Golden State
Number of samples analyzed	25	57	48	50
Coefficient of variation	3.86%	11.7%	18.7%	15.5%
Standard deviation between duplicates	.....	122	87	68.4
Coefficient of correlation DMC–lactic acid	0.828	0.838	0.810	0.844
Regression line	$y = .895x + 181$	$y = 1.337x + 774$	$y = 1.229x + 233$	$y = 1.091x + 230$
Time required	8 hr. for 4 samples	4 hr. for 10 samples	4 hr. for 8 samples	6.5 hr. for 8 samples

[a] y = Lactic Acid (γ/g); x = DMC (millions/g).

The Davidson method gives somewhat higher results than the Ion exchange method. The results of Ion exchange chromatography of a Davidson filtrate are shown in Table 2. These results show that the Davidson method is measuring other substances in addition to lactic acid.

TABLE 2
Fractionation of a Davidson filtrate by Ion exchange chromatography

Fraction	Apparent lactic acid concentration
	(mg/100-g. sample)
Davidson filtrate	118
Cation fraction	4
Anion fraction	88[a]
Nonionic	31

[a] True lactic acid present in this fraction only.

The Golden State method (7) gave somewhat lower results than the Davidson procedure, indicating that it removed interfering materials more efficiently. The extra step made it a longer procedure than the Davidson method.

The Ling method (5), as run here, gave much higher values of apparent lactic acid than any of the three others. This is chiefly because we made no attempt to correct for materials other than lactic acid, which gave a color with ferric chloride in Ling filtrates. Other variations of the Ling procedure (1, 5) call for preparing a calibration curve based on lactic acid originally in the sample (as determined by the Davidson method) plus added lactate. This gives a calibration curve which supposedly corrects for color-producing substances other than lactic acid in the sample. The latter procedure assumes that the amount of interfering material is constant in all milk samples. This assumption is not supported by adequate evidence. The use of the Davidson procedure to determine lactic acid already in the sample also is questionable, since we have shown it to be inaccurate. Therefore, we rejected this procedure and, instead, measured the increase in optical density resulting from added lactate for preparation of the

calibration curve. It may be noted that the method of preparing the calibration curve does not affect the relationship between bacteria counts and apparent lactic acid, although it changes the absolute values of the lactic acid determination.

Even though the Ion exchange method is the only one of the four that approaches a true measure of lactic acid present in the milk samples, we felt that it would be useful to check the correlation of the results obtained by each of the four methods with direct microscopic counts for bacteria. It seemed quite possible that whatever was being measured by the methods which were not specific for lactic acid might be useful in estimating the bacteriological quality of milk. As it turned out, the correlation coefficients between apparent lactic acid and direct microscopic clump counts of bacteria were very nearly the same for all four methods. Therefore, if one is interested in estimating bacterial contamination rather than accurately measuring lactic acid, it is certainly justifiable to use the shortest available method, that described by Ling (5).

Figures 1–4 illustrate graphically the relationship between apparent lactic acid and the direct microscopic counts for bacteria. Lactic acid is plotted against the direct microscopic counts for each of the four methods, and the best possible straight line for the data as calculated by the method of least-squares is shown for each plot. A general and approximately linear increase in lactic acid is observed as the bacteria count increases.

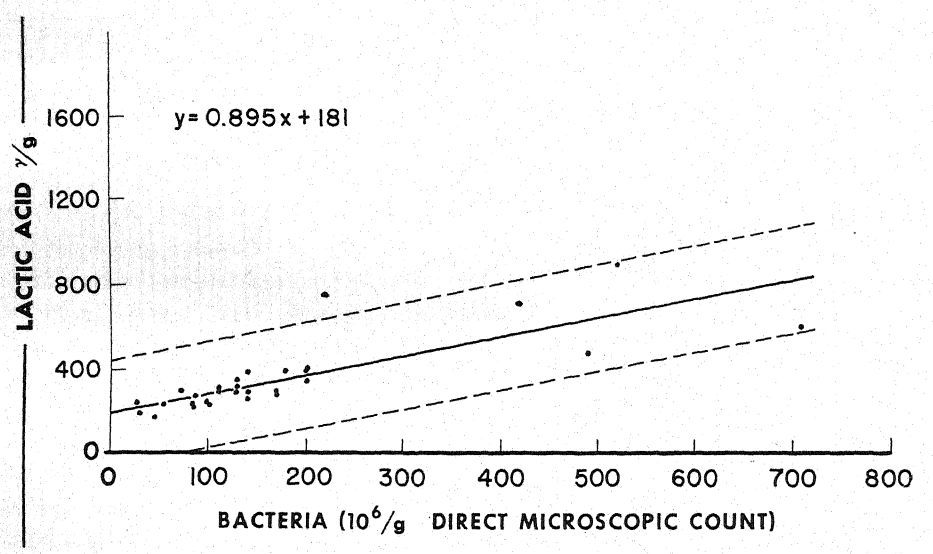


FIG. 1. Lactic acid as determined by the Ion exchange method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

The 95% confidence interval also was calculated (8) and is shown on the graph. This gives the ranges within which 95% of the experimental points may be expected to fall and was calculated to give an idea of the accuracy with which bacteria counts can be estimated from the amount of lactic acid present. For a

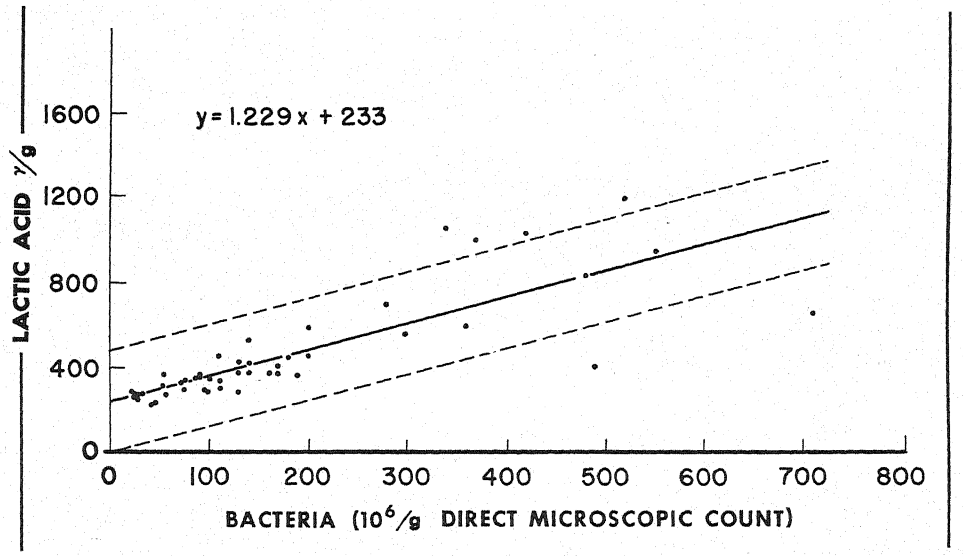


FIG. 2. Lactic acid as determined by the Davidson method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

given amount of lactic acid, the average value of the direct microscopic count may be found on the regression line and the expected range of values is shown by the 95% confidence interval lines. For the Ling method, the confidence interval is ± 192 million; for the Davidson ± 235 million; for the Ion exchange ±245 million; and, for the Golden State method ± 210 million. This very wide range

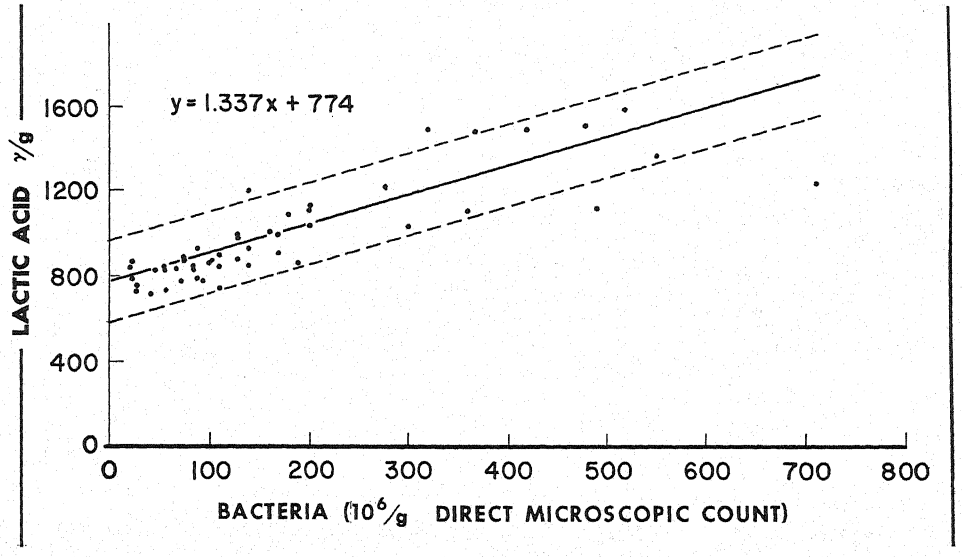


FIG. 3. Lactic acid as determined by the Ling method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

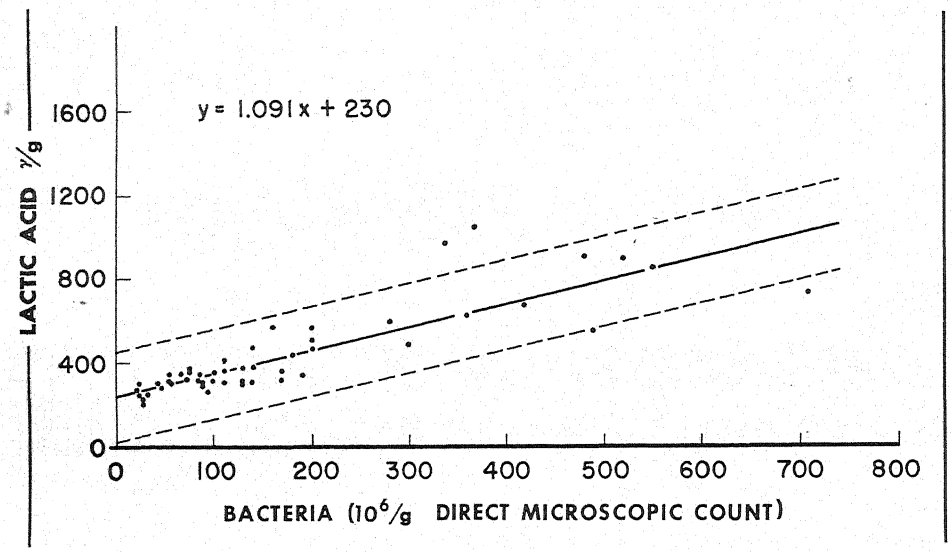


FIG. 4. Lactic acid as determined by the Golden State method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

of direct microscopic counts expected for a given level of lactic acid means that lactic acid content is of limited practical value for estimating numbers of bacteria present.

It is tempting to theorize that much of the observed scatter is due to the inaccuracy of the direct microscopic counts for bacteria. However, the results reported by earlier workers show similar variability (3, 4). Considering the variety of pathways to carbohydrate metabolism of bacteria (10), commonly found in milk, it seems unreasonable to expect better correlation between bacteria counts and lactic acid than is reported here. An additional variable is the formation of various acids including lactic when skimmilk is heated as shown by Morr *et al.* (6). Presumably, the extent of heat treatment would affect the amount of lactic acid from this source, further obscuring any relationship between lactic acid and bacteria counts.

Roller-dried samples gave much higher analyses for lactic acid than reasonably could be expected on the basis of their bacteria counts. The results were,

TABLE 3

Comparison of lactic acid found in spray- and roller-dried samples by the Golden State method

Roller-dried			Spray-dried		
No.	Direct microscopic count	γ/g Lactic Acid	No.	Direct microscopic count	γ/g Lactic Acid
	(millions/g)			*(millions/g)*	
1	13	1,300	1	24	250
2	34	970	2	31	263
3	44	1,075	3	44	220
4	56	1,050	4	56	263

therefore, not included in the above data. Apparent lactic acid content of roller-dried samples and that of spray-dried samples of comparable bacteria count are compared in Table 3. Milk is subjected to much more severe heating in the roller-drying process than in spray drying. The lactic acid in this case probably comes from thermal decomposition of the milk, as previously discussed.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to James Dizikes and others of the Chicago USDA Agricultural Marketing Service dairy laboratory for their cooperation in providing the dry milk samples of known bacterial count used in this study.

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RELATIONSHIPS OF HEAT TREATMENT, SOLIDS-NOT-FAT, AND CALCIUM CHLORIDE TO THE CURD TENSION OF SKIMMILK[1]

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SUMMARY

The effect of high-heat treatments in reducing the curd tension of milk has been further demonstrated. It has also been shown that the curd tension of milk heated as high as 165° F. for 30 min. could be restored, i.e., raised to approximately 54 g. (an average value for milk containing 9% solids-not-fat and heated at 143° F. for 30 min.) by the addition of solids-not-fat and calcium chloride. Calcium chloride concentrations as high as 0.08% gave no detectable off-flavor to the heated skimmilk.

The curd tension of milk has been the subject of numerous investigations, with the expressed purpose of obtaining a milk with less curd tension. This type of milk was considered desirable from a dietary standpoint (3). Decreases in curd tension have been noted as a result of heat treatment (1), the use of pancreatic enzymes (2), dilution with water (3), homogenization (10), and the addition of sodium salts (11). Hill (7) demonstrated that milk from Jersey cows generally had a higher curd tension than milk from Holstein cows. Weisburg *et al.* (12) found that naturally soft-curd milk contained a lower casein concentration and a lower concentration of calcium and phosphorus than did hard-curd milks. They showed that normal variations in the pH and salt balance had no effect on the curd tension value.

The purpose of this study was to determine the effects of various heat treatments on the curd tension of fluid skimmilk and to assess the ability of fortification with nonfat dry milk and calcium chloride to overcome these effects.

EXPERIMENTAL PROCEDURES

Skimmilk was obtained by separating mixed whole milk from the college dairy herd with a cold milk separator. It was stored at 40 ± 2° F. until used. The skimmilk had a solids-not-fat content ranging from 8.8 to 9.1% and a butterfat content of 0.02 to 0.03%. Whenever the solids-not-fat content of the skimmilk was below 9.0%, it was adjusted to 9.0% by fortification with nonfat dry milk to serve as the control milk referred to in this paper. "Lo-Temp"[2] powder was used for fortification. Fortification was done at 95° F., whether before or after heating. Where used, calcium chloride (dihydrate crystal) was added to the heated skimmilk in the form of a 25% aqueous solution, to minimize localized precipitation.

All heat treatments were for 30 min. duration. Since the temperature of treatment was varied, specific temperatures will be given in the section on Results.

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[2] Trademark for a low-heat nonfat dry milk.

The American Dairy Science Association method (4) was used for curd tension determinations. Curd tension measurements were made on a Submarine Signal Company [3] curd tension meter.

EXPERIMENTAL RESULTS

Effect of solids-not-fat. The first step in the study was to screen various high-temperature treatments with the curd tension test, to assess the ability of added nonfat dry milk to overcome the detrimental effects of the heat treatments on curd tension. It was assumed that if any high-temperature treatment lowered the curd tension of milk to such an extent that it could not be overcome by fortification, i.e., raised to a value comparable to curd tension of skimmilk containing 9% solids-not-fat and heated at 143° F. for 30 min., then it probably would not be worth while to study that heat treatment more closely at the present time.

[3] Submarine Signal Company, Boston, Massachusetts.

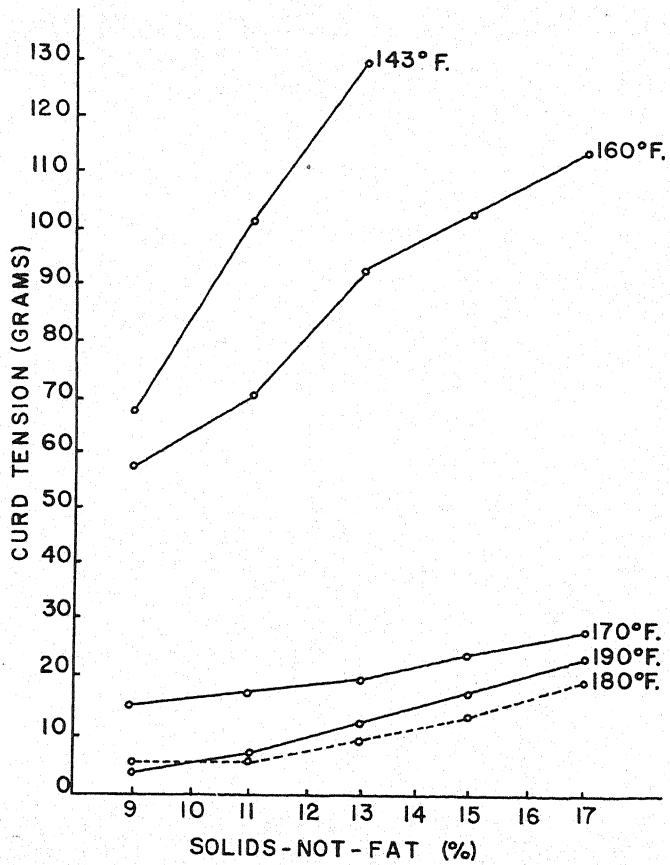


FIG. 1. Effect of heat treatment and solids-not-fat content on the curd tension of skimmilk. Fortification above 13% gave readings out of the range of the machine at 143° F.

The original skimmilk was divided into lots and fortified to give 11, 13, 15, and 17% total solids. These lots of milk were then heat-treated. The results are presented in Figure 1. Each point represents the average of three trials made on different days.

Perhaps the most significant effect shown is the decrease in the curd tension values of milk heated at 170° F. and above, as compared to milk heated at 160° F. for 30 min. Fortification to 17% total solids did not raise the curd tension of the milk heated at 170° F. and above to that of the original milk heated at 143° F.

From this work, it was decided to study heat treatments from 143 to 170° F. for 30 min. duration at closer temperature intervals. Temperature treatments of 143, 150, 155, 160, 165, and 170° F. were used. It was further decided to incorporate the effect of fortifying with nonfat dry milk both before and after heat treatment. In the first set of samples, the original milk was fortified to levels of 9, 10, 11, and 12% solids-not-fat before heat treatment. In the second set of samples, the original milk was heated at the desired temperature, cooled, and then fortified to levels of 9, 10, 11, and 12% solids-not-fat. Curd tension determinations were made on the fortified-heated and heated-fortified skimmilks after they had been stored for 0, 1, and 18 hr. at 40 ± 2° F. The effects of heat treatment, solid-not-fat content, and storage of 40° F. on the curd tension of milk fortified before and after heating, are presented in Tables 1 and 2. The values in both tables are averages of three trials made on different days with a different original skimmilk.

Both tables illustrate the inverse relationship of curd tension to the temperature of heat treatment. On the other hand, the curd tension increased as the

TABLE 1

Effects of heat treatment, solids-not-fat content, and storage on the curd tension of skimmilk fortified before heating

Pasteurization temperature[a]	Hours storage	Curd tension			
		Per cent solids-not-fat			
		9	10	11	12
(° F.)		(g.)			
170	0	8.5	8.8	8.2	9.2
	1	7.5	7.6	7.6	8.0
	18	6.9	7.3	7.3	8.0
165	0	24.0	26.0	28.0	32.5
	1	19.5	23.5	25.0	27.0
	18	18.5	21.0	22.5	26.2
160	0	33.5	40.7	47.2	59.0
	1	30.2	39.0	44.2	54.7
	18	27.5	34.2	39.2	43.2
155	0	47.5	58.2	72.0	86.7
	1	46.5	63.2	72.0	88.7
	18	44.2	56.7	72.0	78.5
150	0	50.5	63.0	75.0	98.0
	1	48.2	63.0	76.2	88.0
	18	44.7	52.2	67.5	76.5
143	0	54.2	67.2	80.0	100.0
	1	51.0	64.2	79.2	91.2
	18	51.0	63.7	79.0	91.0

[a] 30-min. holding time.

TABLE 2
Effects of heat treatment, solids-not-fat content, and storage on the curd tension of skimmilk fortified after heating

Pasteurization temperature[a]	Hours storage	Curd tension			
		Per cent solids-not-fat			
		9	10	11	12
(° F.)		(g.)			
170	0	9.6	12.0	16.6	25.0
	1	8.1	10.0	15.5	22.8
	18	8.1	10.1	15.0	22.3
165	0	21.0	25.0	35.0	43.5
	1	19.0	22.5	29.0	35.0
	18	17.2	21.7	27.0	31.0
160	0	34.7	42.2	52.5	66.2
	1	31.5	38.2	52.2	63.2
	18	28.2	35.0	44.5	56.5
155	0	48.2	59.5	75.7	88.0
	1	50.2	65.2	75.0	91.0
	18	44.2	59.0	66.2	82.0
150	0	53.0	68.5	78.0	95.0
	1	49.5	64.5	74.5	89.7
	18	45.5	60.0	70.2	81.5
143	0	53.5	65.5	79.5	99.7
	1	52.5	60.7	75.7	94.2
	18	51.7	60.7	74.7	93.5

[a] 30-min. holding time.

solids-not-fat content of the milk was increased. Although curd tension dropped when the skimmilk was held for 18 hr., the decrease was not nearly so great as that reported for the elasticity of rennet curd by Hansson and Hietaranta (5). They found that the elasticity (curd resiliency) dropped approximately 75% when the milk was stored at 40° F. for 24 hr.

An important difference between the data presented in the two tables is due to the effect on curd tension of adding nonfat dry milk before and after heat treatment. When milk was fortified before heat treatment (Table 1), that which contained 9% solids-not-fat and was heated at 143° F. for 30 min., had a curd tension of approximately 54 g.[4] at 0 hr. of storage. A comparable curd tension value, or higher, was obtained by heating milk containing 9, 10, 11, and 12% solids-not-fat at 155° F. and milk containing 12% solids-not-fat at 160° F. for 30 min.

More increase in curd tension due to increased increments of solids-not-fat is observed at the higher temperatures when the milk was fortified after heating (Table 2), than was found when the milk was fortified before heating (Table 1). This effect probably is due to the low-heat treatment to which the powder was subjected during its manufacture.

[4] It may be noted that the average curd tension of the original skimmilk in Figure 1 was approximately 70 g. That milk was tested in the fall, whereas the remaining samples were prepared from milk produced in the spring, when cows were on pasture. This probably accounts for the lower average curd tension values obtained. Since subsequent tests showed that acceptable cottage cheese could be made from milk (9% solids-not-fat heated at 143° F. for 30 min.) with a curd tension of approximately 54 g., this curd tension value was accepted as the standard.

There is very little difference in the curd tension at 0 hr. aging of milk of 9% solids-not-fat heated at 143 and 150° F. Comparable curd tension values (53 g.), or higher, are evident at 155° F. for milk containing 10, 11, and 12% solids-not-fat, and 160° F. for milk containing 11 and 12% solids-not-fat.

Effect of calcium chloride. The literature contains contradictory statements as to the effects of using calcium chloride to overcome the detrimental effects of heat treatment. The effects of calcium chloride on the curd tension of fresh skimmilk fortified to 9, 10, 11, and 12% solids-not-fat, both before and after heat treatments of 143, 150, 155, 160, 165, and 170° F., were determined. Calcium chloride (25% aqueous solution) was then added to the aliquots of milk, to give concentrations of 0.00, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.35% added calcium chloride. The mixture was shaken three different times during a 15-min. period before its curd tension was determined.

Figure 2 shows typical results obtained when varying levels of calcium chloride were added to milk heated at temperatures ranging from 143 to 170° F. In all cases, there was a maximum curd tension obtained with serial additions of calcium chloride, the maximum occurring at different levels for different

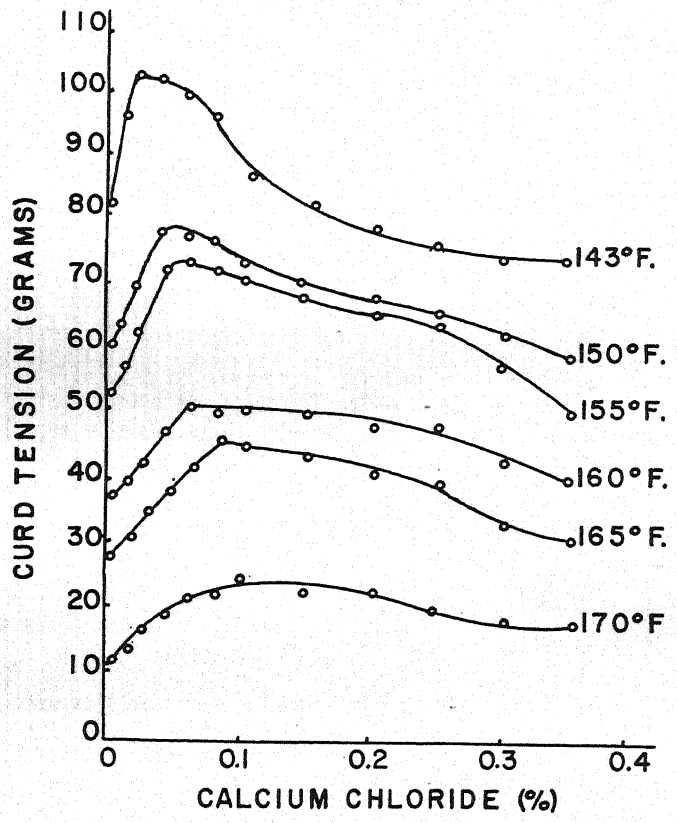


FIG. 2. The effect of calcium chloride on the curd tension of milk fortified before heating to 12% solids-not-fat and heated at the specified temperatures for 30 min.

treatments. The maximum curd tension values for all solids levels and heat treatments studied are reported in Tables 3 and 4. The curd tension values for milk of all solids levels heated at 160° F. are high in relation to the pattern set forth by the other heat treatments. As can be observed from the standard deviations associated with the average curd tension values, there was considerably more variability associated with the curd tension data at 160° F. than at the other temperatures. No explanation for this is evident.

Table 3 illustrates the direct relationship between heat treatment and the amount of calcium chloride required to produce a maximum curd tension in milk fortified before heating. As the heat treatment was increased, the amount of calcium chloride required also increased. For milk heated at 143° F. for 30 min., approximately 0.02% calcium chloride was necessary to produce a maximum curd tension, whereas 0.10% was necessary in milk heated at 170° F. for 30 min. However, the amount of calcium chloride required to produce a maximum curd tension remained the same for milk at a given heat treatment with varying solids-not-fat levels.

Table 4 shows that the amount of calcium chloride necessary to produce a maximum curd tension in milk fortified after heating is approximately the same as for milk fortified before heating (Table 3), and again varied directly with heat treatment but remained the same for milk with varying solids-not-fat content at a given heat treatment.

TABLE 3

Maximum curd tension obtained by the addition of calcium chloride to milk fortified before heating

Pasteurization temperature[a]	Solids-not-fat	$CaCl_2$ giving maximum curd tension	Maximum curd tension	Variation in curd tension
(° F.)	*(%)*	*(%)*	*(g.)*	*(standard deviation)*
170	9			
	10			
	11			
	12	0.10	35.0	2.36
165	9			
	10			
	11	0.08–.10	37.3	2.95
	12	0.08–.10	44.3	1.77
160	9	0.08	52.0	8.28
	10	0.08	62.6	10.65
	11	0.08–.10	76.0	8.86
	12	0.08–.10	81.6	7.68
155	9	0.06–.08	45.6	1.18
	10	0.06–.08	56.6	1.18
	11	0.06–.08	66.6	1.77
	12	0.06–.08	72.3	2.36
150	9	0.04–.06	53.6	1.18
	10	0.04–.06	56.0	2.36
	11	0.04–.06	66.3	1.77
	12	0.04–.06	77.6	2.96
143	9	0.02–.04	65.6	0.59
	10	0.02–.04	69.3	1.77
	11	0.02	81.0	1.18
	12	0.02–.04	101.6	2.96

[a] 30-min. holding time.

TABLE 4

Maximum curd tension obtained by the addition of calcium chloride to milk fortified after heating

Pasteurization temperature[a]	Solids-not-fat	$CaCl_2$ giving maximum curd tension	Maximum curd tension	Variation in curd tension
(° F.)	*(%)*	*(%)*	*(g.)*	*(standard deviation)*
170	9			
	10			
	11	0.10	32.6	2.95
	12	0.10	40.6	1.18
165	9			
	10	0.08–.10	41.6	1.77
	11	0.08–.10	56.6	1.77
	12	0.08–.10	63.6	1.18
160	9	0.08	52.3	4.71
	10	0.08	65.6	7.11
	11	0.08	79.3	10.04
	12	0.08	89.0	5.32
155	9	0.06–.08	44.6	2.95
	10	0.06–.08	58.0	3.55
	11	0.06–.08	71.0	1.18
	12	0.06–.08	72.3	6.50
150	9	0.04–.06	53.3	2.96
	10	0.04–.06	65.3	2.96
	11	0.04–.06	77.3	3.55
	12	0.04–.06	95.0	2.36
143	9	0.02–.04	67.3	2.36
	10	0.02–.04	73.0	1.18
	11	0.02–.04	84.6	1.18
	12	0.01–.04	108.6	9.45

[a] 30-min. holding time.

The data presented in Tables 3 and 4 indicate that calcium chloride can be utilized to help overcome the detrimental effects of heat treatment on curd tension. Milk fortified to 11 and 12% solids-not-fat and heated at 160° F. for 30 min., produced a curd tension value well above 54 g. when calcium chloride was used. For milk fortified after heating to 11 and 12% solids-not-fat, the use of optimum amounts of calcium chloride permitted heating as high as 165° F. for 30 min. without reducing the curd tension below 54 g.

Three judges tasting independently were able to detect a slight salty flavor in the milk at the highest concentration of calcium chloride reported in this paper (0.10%), but were not able to detect any off-flavor at a lower concentration attributable to the addition of calcium chloride.

DISCUSSION

It has been confirmed that the curd tension of skimmilk that had been heated excessively could be restored by increasing the concentration of soilds-not-fat and by adding calcium chloride. This was expected, since it is well known that increasing concentrations of casein and calcium favor strong curd character. The primary objective of this paper was to determine the maximum heat treatment to which skimmilk could be subjected and its original curd tension subsequently restored by the addition of solids-not-fat and calcium chloride. Milk

containing 9% solids-not-fat and heated to 143° F. for 30 min. had a curd tension value of 54 g. and was taken as the standard.

When the solids-not-fat level was raised to 10% or above, it was immaterial whether fortification was done before or after heat treatment at 150 and 155° F., because the benefits of fortification on curd tension exceeded the detrimental effects of heat.

More calcium was needed to produce a maximum curd tension as the heat treatment was increased. This could be partially explained as a restoration of precipitated calcium salts (6), or on the basis that the milk did not contain the optimum amount of calcium for maximum curd formation.

The fact that skimmilk can be heated to temperatures at which the curd tension can not be restored by added solids-not-fat and calcium, might indicate that denatured serum proteins interfere physically with normal curd formation or form complexes that retard or reduce curd strength. Rudnick (8) favored the former explanation.

The maximum heat treatment to which milk was subjected and the curd tension restored to approximately its original value (54 g.) was 165° F. for 30 min.

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EFFECT OF HARD WATER ON THE HEAT STABILITY OF SKIMMILK POWDER[1]

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SUMMARY

Protein stability in reconstituted skimmilk is adversely affected by high calcium and magnesium levels in the reconstituting water. The heat stability, as measured by the Storrs stability test, is a function of total hardness, and is not changed by varying the calcium-to-magnesium ratio. The effect of magnesium ions can be balanced by sequestration with equivalent quantities of sodium hexametaphosphate, but with calcium more than equivalent quantities of this sequestering agent are needed. Sequestration of a portion of the hardness of very hard waters is suggested as a possible method for avoiding premature protein coagulation during heat processes used in the manufacture of cottage cheese and in the preparation of media for propagation of cheese starters.

When very hard water is used to reconstitute skimmilk powder, premature coagulation of proteins in the milk occurs during certain heat treatments. In some areas in the Province of Ontario, where the hardness in water exceeds 1,000 p.p.m., this problem has been encountered. Figure 1 shows a typical example of the effect of one heat treatment on milk reconstituted with water. Preliminary experiments indicated that the heat stability of the dry milk is inversely proportional to the degree of hardness in the reconstituting water; therefore, the effects of calcium and magnesium ions on heat stability were investigated, both separately and together, using the Storrs heat stability test as an index of stability.

PREPARATION OF SYNTHETIC HARD WATER SOLUTIONS

Stock calcium and magnesium solutions were prepared from appropriate quantities of calcium chloride, magnesium chloride, and magnesium sulphate. Four series of synthetic hard waters, each including hardness levels of 0, 50, 100, 200, 400, and 800 p.p.m. (calculated as $CaCO_3$) were made by diluting the stock solutions with distilled water. One series contained only calcium, one contained only magnesium, and the other two contained both calcium and magnesium in the ratios 2:1 and 1:6. The latter ratio was selected to obtain a synthetic hard water in which the magnesium ion content was much in excess of the calcium ion content. The hardness levels of these solutions were checked by the method of Betz and Noll (1), using Eriochrome Black T as a total hardness indicator, and Murexide as a calcium indicator.

To determine the effect of sodium hexametaphosphate on the destabilizing influence of calcium and magnesium ion, synthetic hardness solutions were made up by mixing the stock hardness solutions with stock hexametaphosphate solution and distilled water. The calcium and magnesium equivalence of the hexameta-

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[1] This is the legal name of this product in Canada.

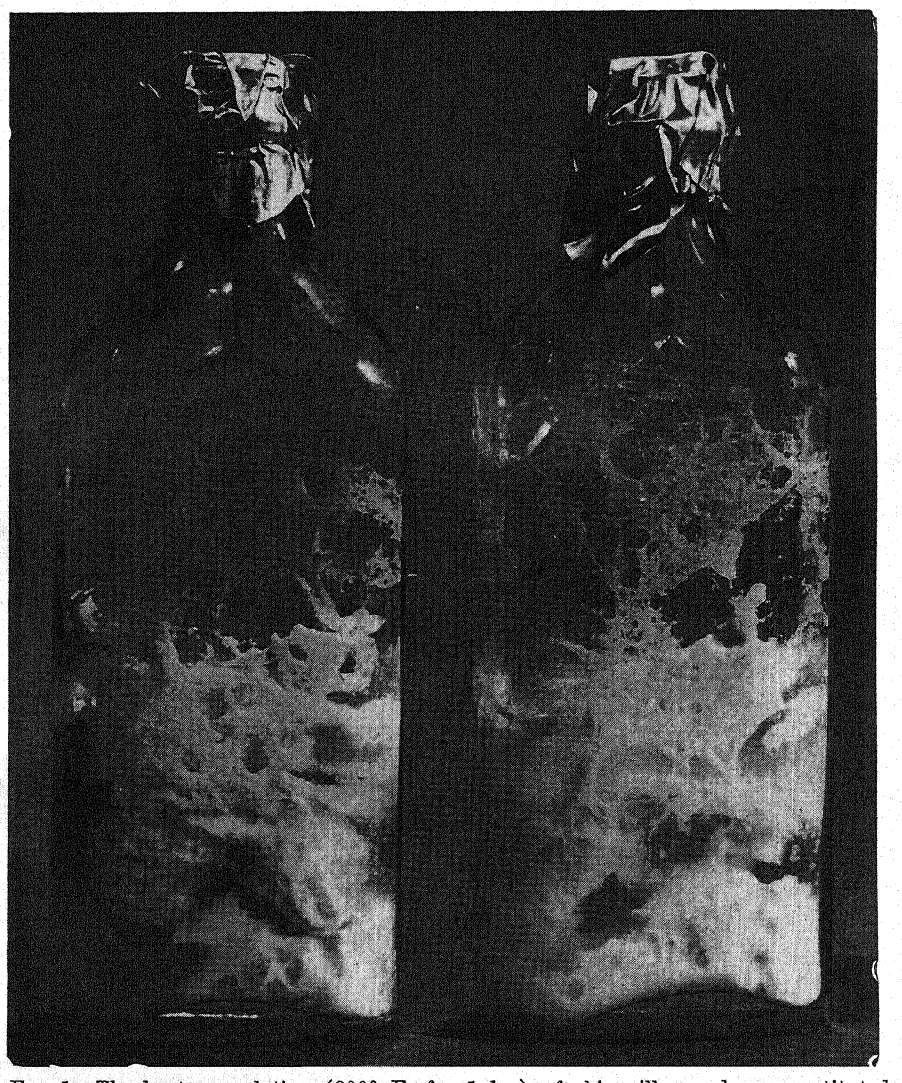


FIG. 1. The heat coagulation (200° F. for 1 hr.) of skimmilk powder reconstituted in extremely hard natural water obtained from a local cheese factory.

phosphate solution was determined by using it in place of EDTA in Betz and Noll titrations of the calcium and magnesium stock solutions. Sequestration levels of 25, 50, 75, and 100% indicate the respective percentage of an equivalent quantity of hexametaphosphate that was used in preparation of the synthetic hard waters.

EXPERIMENTAL PROCEDURES AND RESULTS

Reconstituted skimmilk samples were prepared by mixing 24.72% g. of a low heat powder with 200 ml. of synthetic hard water, using a Waring Blender operated at high speed for 10 min. The milk samples thus prepared (about

11% total solids) were then stored for 12 hr. at 48° F., after which they were tested for heat stability by the method of Storrs (2).

A comparison of the Storrs stability numbers of milks reconstituted from two different low heat powders (Samples 1 and 2) at various hardness levels, but with no sequestering agent added, is shown in Figure 2. The Storrs stability

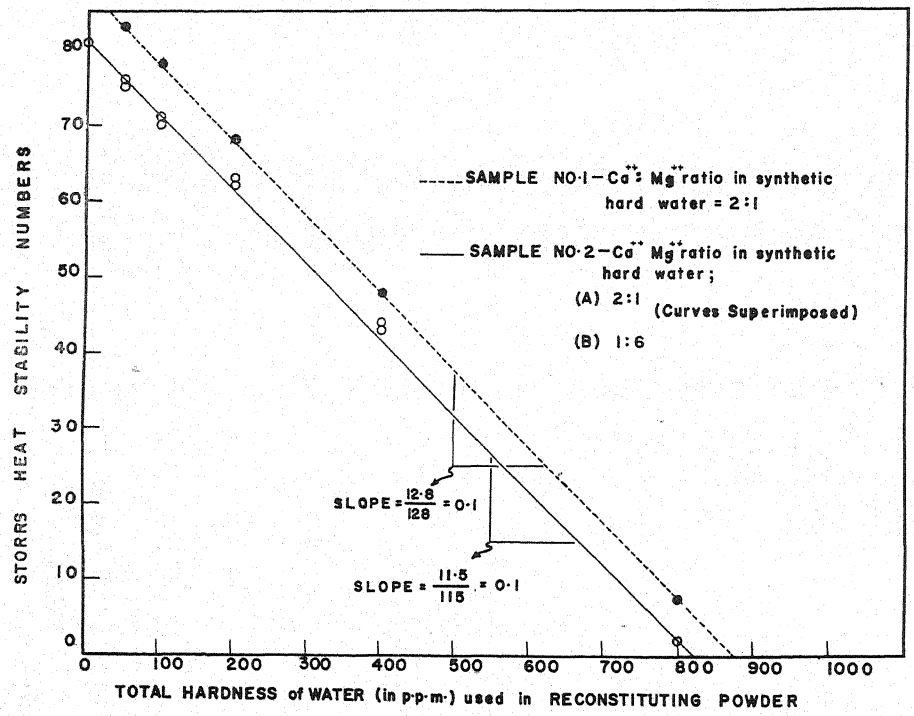


FIG. 2. The Storrs heat stability test on two different samples of low heat skimmilk powder, showing the effect of the Ca^{++} to Mg^{++} ion ratio on the heat stability of the milk.

number was inversely proportional to the degree of total hardness of the reconstituting water. Hardness levels of 800 to 900 p.p.m. reduced the stability number to zero. The two powders produced parallel curves. When the calcium to magnesium ratio was changed from 2:1 to 1:6 with Powder Sample No. 2 (Figure 2), and when magnesium (zero per cent sequestration curve in Figure 3) or calcium (zero per cent sequestration curve in Figure 4) was used alone, identical curves were produced. This indicated that the destabilizing effects of calcium and magnesium ions are the same in the concentration range studied.

The addition of percentages of an equivalent quantity of sodium hexametaphosphate to magnesium hardness solutions produced the same percentage of stability recovery in the reconstituted milks (Figure 3). When 100% of the equivalent quantity of sequestering agent was added, the resultant reconstituted milks had the same stability number as milk reconstituted with distilled water.

The addition of percentages of an equivalent quantity of sodium hexameta-

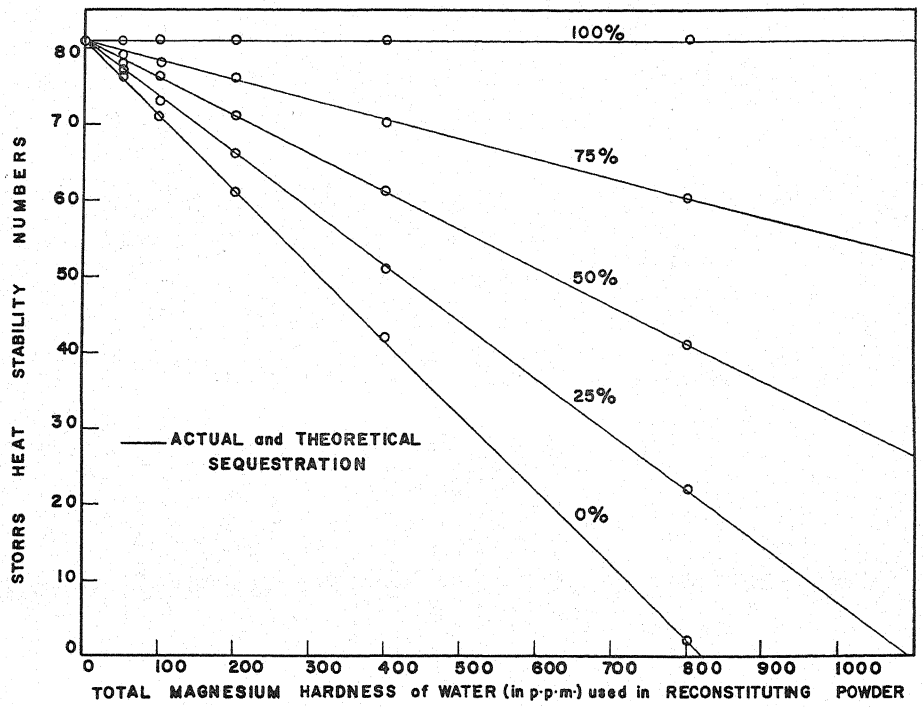


FIG. 3. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for Mg^{++} ions.

phosphate to calcium hardness solutions resulted in less than the theoretical recovery (Figure 4). Thirty-three per cent more than an equivalent quantity of hexametaphosphate was required for complete stability recovery, and in the 800 p.p.m. hardness solution this quantity of sequestering agent failed to provide as much recovery as that achieved by the addition of only 25% of an equivalent amount.

The stability recovery achieved by addition of hexametaphosphate to hard water solutions containing both calcium and magnesium is shown in Figure 4. As would be expected, the percentage stability recovery here also is less than the percentage of an equivalent quantity of added hexametaphosphate.

An attempt was made to protect reconstituted milk made with synthetic hard water against destabilization during each of two heat treatments commonly used in the preparation of cultures for the manufacture of cottage cheese. The heat treatments used were 180° F. for 1 hr. and 245° F. for 15 min. Milks reconstituted from 800 p.p.m. hardness water containing various percentages of an equivalent quantity of hexametaphosphate were subjected to the heat treatments and were then examined for evidence of coagulation. An additional set of samples containing EDTA in place of hexametaphosphate was treated in the same way. Milk samples containing no sequestering agent coagulated during both heat treatments. The addition of EDTA equivalent to 25% or more of

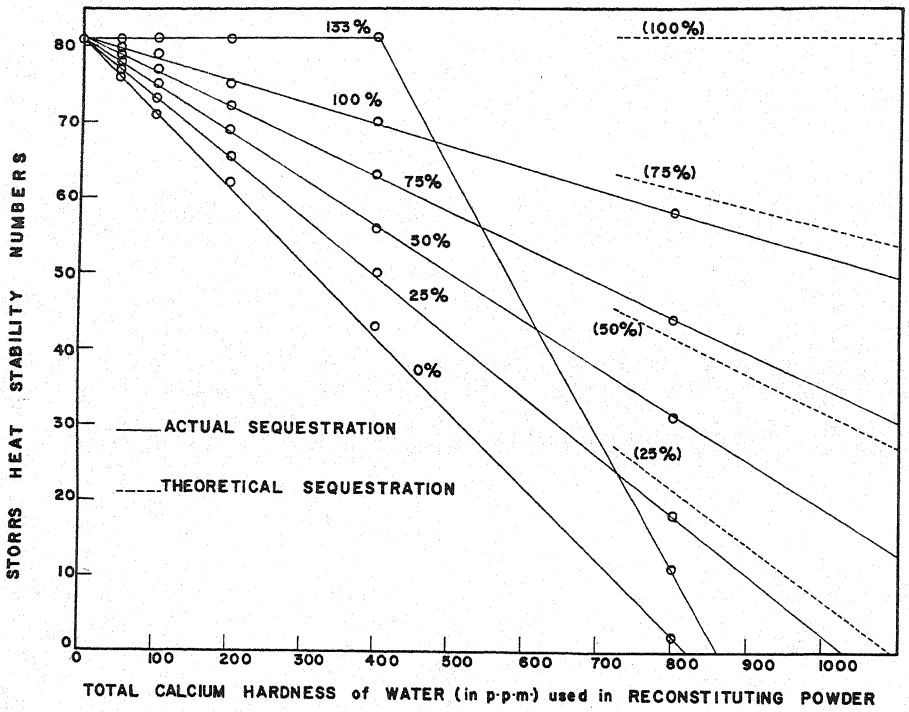


FIG. 4. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for Ca^{++} ions.

800 p.p.m. of hardness prevented coagulation during both heat treatments. The addition of hexametaphosphate equivalent to 25% or more of the 800 p.p.m. of hardness protected the milk against coagulation during the 180° F. hour treatment, but provided no protection against the 245° F.–15 min. treatment.

DISCUSSION

The Storrs stability data shown in Figures 2, 3, 4, and 5 indicate that the heat instability of reconstituted powders is caused by the presence of excess calcium or magnesium ions. It is reasonable to expect that the stability of milk in which agents are used to sequester these excess ions would depend upon the heat stability of the complexes formed. The calcium and magnesium complexes formed by EDTA are known to be more heat stable than those formed by hexametaphosphate. The relative stabilities of these complexes are illustrated by the results of attempts to prevent coagulation during sterilizing treatments. Skimmilk powder reconstituted in hard water treated with sodium hexametaphosphate proved to be unstable when subjected to a laboratory sterilizing process (245° F. for 15 min.); whereas, the same powder reconstituted in water treated with EDTA showed no instability during the same heat treatment. Both reconstituted powders were stable when subjected to the less severe plant sterilizing treatment (180° F. for 1 hr.).

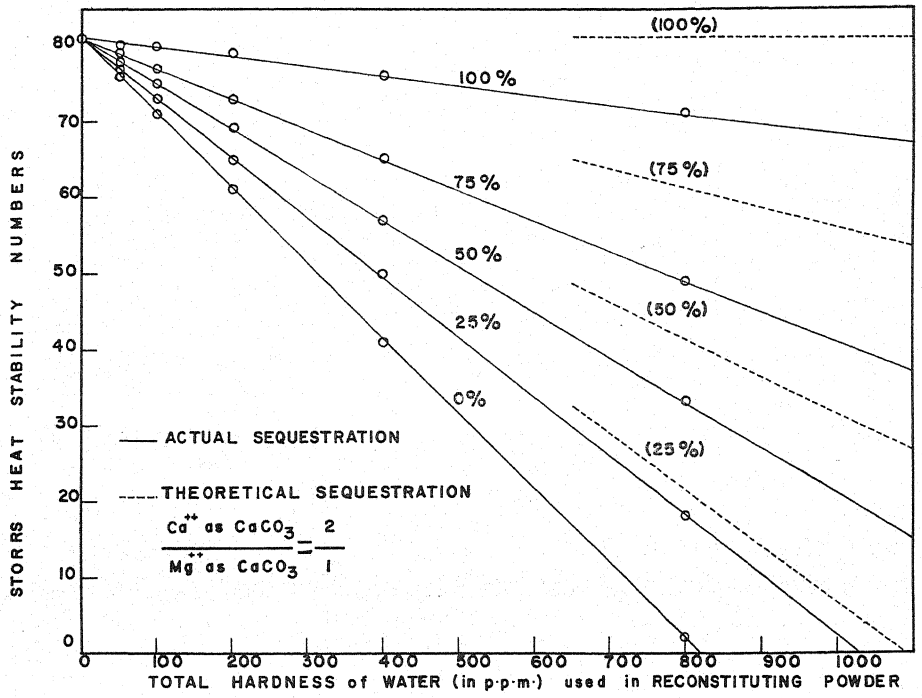


FIG. 5. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for Ca^{++} and Mg^{++} ions.

The experimental results indicate that the stability of a reconstituted powder may be improved in some heat treatments by the addition of sodium hexametaphosphate to the water used in reconstitution. By determining the Storrs heat stability number of the skimmilk powder in question, and the hardness at which instability of the reconstituted milk occurs, subsequent calculation may be made of the minimum amount of sodium hexametaphosphate required to prevent the heat coagulation of the milk in the existing heat treatment.

Before employing sodium hexametaphosphate to prevent the heat coagulation of reconstituted milk, it would be advisable to consult local health authorities on the legality of its use.

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FACTORS INFLUENCING THE METABOLIC ACTIVITY OF BOVINE SPERMATOZOA. V. SEASON

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SUMMARY

The aerobic metabolic activity (oxygen consumption, fructose utilization, and lactic acid accumulation) of semen and washed spermatozoa from collections made from eight bulls during two seasons (winter and summer) and 2 yr. was determined in two diluents and three treatments. The treatments included the suspension of washed cells in diluent containing fructose or diluted seminal plasma from the same ejaculate or that from the same bull stored from the previous season. Summer-collected cells lived through the 4-hr. incubation at 37° C. better, consumed more oxygen, and accumulated less lactic acid whether incubated as semen or as washed cells resuspended in fructose or in their own diluted seminal plasma. Diluted seminal plasma of the previous season reversed some of these trends. The primary though not exclusive seasonal effect in metabolism resides in the cells.

In many of the vertebrates, spermatogenesis is a cyclic phenomenon. Although spermatozoa are produced throughout the year in the bull, some seasonal fluctuations in spermatogenesis and fertility exist. For example, Erb *et al.* (4) found in the Purdue herd that the highest conception rate occurred in May, and the lowest in August; while in Louisana, the conception rate was reported by Seath and Staples (11) to be lower during the summer months than in any other period of the year.

Evidence indicates that temperature, light, relative humidity, and possibly other atmospheric factors influence the quality of the semen produced by the bull (3). Under controlled conditions, the continuous exposure of young dairy bulls to environmental temperatures exceeding 85° F. for periods longer than 5 wk. was reported to impair spermatogenesis (2). Other studies have indicated that light also may be an important factor. Mercier and Salisbury (6, 7) studied the conception rate of naturally bred cattle in Eastern Canada and the artificially bred cattle of New York State. Temperature changes at that latitude did not measurably affect the fertility level of the cattle. However, the length of daylight was directly related to the conception rate.

If part of the variations in fertility which occur with the seasons of the year can be attributed to the male, it is reasonable to expect that changes in biochemical behavior of the male reproductive cells might accompany the observed variations. Smith *et al.* (12) observed a significant stimulation of the succinate dehydrogenase activity, on washing, of bovine spermatozoa obtained during the winter and spring months. On the other hand, no significant activation of the succinate dehydrogenase occurred on washing of spermatozoa obtained during the summer and fall months.

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In an earlier examination of data obtained in this laboratory, the aerobic fructolysis of bovine semen collected during the winter was significantly greater than that collected in the summer months (10). Since this evidence was obtained from studies having other objectives, an experiment was designed to clarify the observation. In addition, the study was designed to determine whether the metabolic responses were due to the cells being different from season to season or to a difference in the seminal plasma.

EXPERIMENTAL PROCEDURE

Semen specimens were collected from eight bulls during the winter and summer seasons of 2 yr. To represent the extremes in environmental conditions of temperatures and daylight hours, ejaculates were collected during the months of December to February for the period designated as winter, and during July and August for the summer. The mean maximum and minimum temperatures for the first and second winters were 30.8 and 13.9° F. and 36.7 and 20.4° F.; the daylight hours were 10.2 and 9.5 for the periods of collection during each of the two winters. The mean maximum and minimum temperatures for the two summers were 88.9 and 67.5° F. and 81.5 and 62.4° F.; the daylight hours were 14.9 and 14.9, respectively.

Two ejaculates were collected from each of eight bulls at intervals of two to eight days during the winter of 1954-55, summer of 1955, winter of 1955-56, and summer of 1956, making a total of 64 ejaculates. Aliquot specimens were suspended in two diluents, saline and phosphate buffer, which were subjected to three treatments: untreated diluted semen, twice-washed spermatozoa suspended in fructose diluent, and twice-washed spermatozoa suspended in diluted seminal plasma from the same ejaculate. Forty-eight of these 64 ejaculates were subjected to an additional treatment, i.e., specimens of the twice-washed cells were resuspended also in diluted (saline and phosphate buffer) seminal plasma obtained during the preceding season. Thus, there was a total of 480 observations in the entire experiment.

Semen specimens and diluents, physiological saline and sodium phosphate buffer, were prepared in the manner described previously (10). Briefly, each semen sample was divided into three portions, A, B, and C. Portion A remained untreated, B was washed twice with phosphate buffer (pH 7) and aliquot specimens were suspended in phosphate-fructose solution, seminal plasma (which consisted of 0.6 ml. phosphate buffer and 0.2 ml. seminal plasma that was removed prior to the washing of the cells), and seminal plasma (in similar proportions) from the same bull of the previous season (stored at 0° F.). Portion C was treated as in B, but the sodium phosphate buffer solution was replaced with physiological saline (0.9% NaCl).

Spermatozoa suspended in a medium free from seminal plasma were obtained by centrifuging whole semen at approximately 400 × gravity for 10 min. and removing the supernatant seminal plasma. The sperm cells were resuspended in the diluting solution to the original volume of the ejaculate. The suspension was again centrifuged, the supernatant removed, and the cells resuspended in fresh diluent. The process was repeated a second time.

In subsequent dilutions of the cells, physiological saline was added to those cells washed with saline, and phosphate buffer was added to the phosphate-washed cells.

Respiration was measured by the direct method of Warburg (13) at 37° C. for 4 hr. in 5-ml. single-side-arm reaction flasks. Carbon dioxide was absorbed with 20% KOH (0.1 ml.) placed in the center well. Two-tenths of a milliliter of semen or spermatozoa suspension containing approximately 3×10^8 cells was placed in the side arm and added to 0.8 ml. of diluent in the main compartment after an equilibration period of 10 min. Aliquot specimens were taken in the beginning and end of each experiment for fructose (5, 9) and lactic acid (1) determinations.

When the study was initiated, seminal plasma of semen collected during the seasonal period preceding the beginning of the study was not available. Thus, three seasons (one winter and two summers) were represented for the treatment involving the resuspension of twice-washed spermatozoa in seminal plasma of the preceding season.

The bulls were fed hay and University Grain Mix 38 and were allowed to exercise daily in a dry lot, but were not pastured.

RESULTS

Treatment I—diluted whole semen. The summary of the average aerobic metabolic responses of diluted semen collected during the two summers and two winters is given in Table 1. The percentage of motile cells immobilized during the 4-hr. incubation period in saline was lower than it was in phosphate buffer. The respiratory activity of semen diluted in physiological saline was approximately twice as great as that diluted in phosphate buffer. Fructose uptake and lactic acid accumulation were generally lower in saline diluent than in phosphate buffer. These responses were consistent throughout the study.

The percentage of motile cells immobilized during the incubation period was slightly lower for the summer-collected cells than for the winter-collected cells. When the seasonal fructolytic responses were compared, winter-collected semen respired less but utilized more fructose and accumulated more lactic acid

TABLE 1

Summary of the aerobic metabolism of semen

Diluent	Seasons of collection	Motility				Aerobic activity			$+Z_{lactate}$
		Initial		Final		$-Z_{O_2}$	$-Z_{fructose}$	$+Z_{lactate}$	$-Z_{fructose}$
		%	Rate	%	Rate	μl.	μg.	μg.	%
NaCl	Winter	53.2	3.2	40.5	2.2	9.1	42.4	17.9	42
	Summer	61.0	3.4	48.2	2.7	9.9	35.6	6.4	18
HPO_4	Winter	52.8	3.2	24.1	1.1	5.0	47.0	27.3	58
	Summer	61.0	3.4	35.4	2.0	5.2	35.2	24.4	69

Mean of 32 observations.
Mean cell count: Winter, 263.6×10^6 cells/ml of diluent.
Summer, 285.5×10^6 cells/ml of diluent.

than summer-collected semen. Though the differences were not statistically significant, the seasonal differences with these unwashed spermatozoa in semen were similar to the earlier results (8) under the same conditions.

The individual seasonal metabolic responses of semen are shown in Table 2. The mean initial motility of the semen samples of the second winter was approximately 9% higher than that of the samples collected during the first winter, although greater fructose uptakes were encountered with the semen of the first winter. Differences in the metabolic activity of the two winters were not significant.

TABLE 2

Seasonal aerobic metabolic activity of semen

Seasons of collections	Diluent	Motility				Aerobic activity			$+Z_{lactate}$
		Initial		Final		$-Z_{O_2}$	$-Z_{fructose}$	$+Z_{lactate}$	$-Z_{fructose}$
		%	Rate	%	Rate	µl.	µg.	µg.	%
1st winter	NaCl	50.7	3.2	39.7	2.2	9.4	50.7	13.7	27
	HPO_4			24.1	1.2	4.9	55.0	31.3	57
2nd winter	NaCl	55.6	3.2	41.3	2.2	8.8	34.2	22.1	65
	HPO_4			24.4	1.0	5.2	38.9	23.3	60
1st summer	NaCl	56.9	3.4	43.8	2.8	10.1	28.9	1.7	6
	HPO_4			30.1	2.1	5.6	29.7	23.9	80
2nd summer	NaCl	65.0	3.5	52.5	2.6	9.7	42.4	11.2	26
	HPO_4			40.6	1.8	4.8	40.8	24.8	61

Mean of 16 observations.
Mean cell count: 1st winter, 272.6×10^6 cells/ml of diluent
2nd winter, 254.7×10^6 cells/ml of diluent.
1st summer, 305.0×10^6 cells/ml of diluent.
2nd summer, 266.0×10^6 cells/ml of diluent.

In comparing the summer seasons, the mean initial motility was approximately 12% higher for the semen samples collected in the second than in the first summer, and the fructose uptake and lactic acid accumulations were higher for the second summer. However, the degree of variation in respiration, substrate uptake, and lactate accumulation was large, and the differences noted between the two summers were not significant.

Treatment II—twice-washed spermatozoa diluted in fructose solution. In the phase of the experiment just discussed (Treatment I), the fructolytic activity of winter-collected semen was greater than that of summer-collected semen. However, when spermatozoa from these same ejaculates were washed and resuspended in fructose diluent, summer-collected spermatozoa were more active metabolically than winter-collected spermatozoa.

About 58% of all the winter-collected cells were immobile after the 4-hr. incubation period, and 37% for the summer-collected spermatozoa (Table 3). Respiration and fructose uptake of summer-collected spermatozoa were significantly ($P < 0.01$) greater than winter-collected cells. Lactic acid accumulation, though greater in the summer-collected cells, was not significantly ($P > 0.05$) different. These responses of washed cells resuspended in fructose diluent differed from the aerobic glycolysis of spermatozoa in semen. The differences in the aerobic gly-

TABLE 3
Aerobic metabolic behavior in fructose solution of twice-washed spermatozoa

Seasons of collection	Diluent	Mean cell count $\times 10^6$	Motility				Aerobic activity			$+Z_{lactate}$
			Initial		Final		$-Zo_2$	$-Z_{fructose}$	$+Z_{lactate}$	$-Z_{fructose}$
			%	Rate	%	Rate	µl.	µg.	µg.	%
Winter	NaCl	254.4	48.2	2.8	31.2	1.4	8.2	19.8	6.0	30
	HPO_4	248.8	42.7	2.4	6.8	0.4	4.1	18.8	17.1	91
Summer	NaCl	359.9	52.8	3.1	39.0	2.0	9.0	22.0	6.8	31
	HPO_4	344.4	50.0	2.8	25.9	1.2	4.7	25.0	19.5	78

colysis of semen and washed cells resuspended in fructose diluent suggest that summer-collected semen was inherently as active in their fructolytic processes as winter-collected semen. The difference in behavior of these two seasonally grouped cells may be due either to the seminal plasma or to the removal of a component or components from the cells by washing.

Treatment III—twice-washed spermatozoa resuspended in the plasma of the same ejaculate. The responses of twice-washed spermatozoa resuspended in diluted seminal plasma of the same ejaculate are summarized in Table 4. The

TABLE 4
Effect of diluent and seminal plasma (plasma of the same ejaculate) on twice-washed spermatozoa

Seasons of collection	Diluent	Mean cell count $\times 10^6$	Motility				Aerobic activity			$+Z_{lactate}$
			Initial		Final		$-Zo_2$	$-Z_{fructose}$	$+Z_{lactate}$	$-Z_{fructose}$
			%	Rate	%	Rate	µl.	µg.	µg.	%
Winter	NaCl	251.2	48.4	2.8	42.6	2.4	9.6	27.8	19.4	70
	HPO_4	241.8	42.5	2.4	26.1	1.2	5.6	23.5	41.6	177
Summer	NaCl	359.9	52.8	3.1	50.4	3.0	9.9	28.8	16.6	58
	HPO_4	344.4	50.0	3.0	40.0	2.3	5.6	32.2	31.4	98

decrease in motility of winter- and summer-collected cells during the test period was about 24 and 10%, respectively. Respiration of winter- and summer-collected cells was approximately the same and was not statistically ($P > 0.05$) different. The fructose loss in summer-collected samples was approximately 16% more than in winter-collected spermatozoa, but the difference was not significant. On the other hand, the lactic acid accumulated in summer-collected samples was significantly ($P < 0.01$) lower than in winter cells. This lower accumulation of lactate by summer-collected, twice-washed spermatozoa is similar to that observed in semen, but contrary to that observed in twice-washed cells provided with fructose as a sole substrate. This result suggests that some entity, which may have been present or absent in the seminal plasma obtained during the summer months, depressed the lactic acid accumulation of twice-washed, summer-collected cells.

Treatment IV—twice-washed spermatozoa resuspended in the plasma collected during the preceding season. To test whether seminal plasma influenced the seasonal metabolic pattern of bovine spermatozoa, cells from a bull, collected

during one season, were washed and resuspended in seminal plasma removed from semen collected in the preceding season from the same animal and frozen until used. Seminal plasma was not available from the summer season preceding the first winter of the study, but the data of the three subsequent seasons are presented in Table 5.

The percentage of winter-collected cells suspended in winter plasma (plasma of the same ejaculate) immobile at the end of the incubation period was approximately 24%, and winter-collected cells in summer plasma (plasma of the same bull but of the preceding season, or stored plasma) was 33%. The percentage of summer-collected washed cells suspended in summer plasma (plasma of the same ejaculate) immobilized during the incubation period was approximately 12%, and of summer-collected cells suspended in winter (stored) plasma, 18%.

The respiration, fructose uptake, and lactic acid accumulation (Tables 5 and 6) were not significantly different ($P > 0.05$) when spermatozoa collected in winter were twice-washed and resuspended in winter plasma (plasma of the same ejaculate), or in summer (stored) plasma. When summer cells washed and suspended in winter (stored) plasma were compared to those suspended in summer plasma (plasma of the same ejaculate), the fructose uptake was significantly ($P < 0.05$) greater. Respiration and lactic acid accumulation did not differ stastistically. When the activity of the cells collected in winter was compared to that of summer samples, regardless of the treatment of diluents, fructose uptake ($P < 0.01$) and lactic acid accumulation ($P < 0.05$) were greater in the summer cells.

DISCUSSION

As reported earlier (10), and irrespective of treatments, the livability of the spermatozoa suspended in saline was better at the end of the incubation period than of those diluted in the phosphate buffer. Respiration of saline-diluted sperm cells was approximately twice as great as it was in phosphate diluent, but fructose uptake and lactic acid accumulation were lower. These responses were generally consistent throughout this study.

In the seasonal comparisons of the survival of the spermatozoa, whether in diluted semen, washed cells suspended in fructose diluent, or washed cells suspended in diluted seminal plasma of the same ejaculate, the percentage of motile spermatozoa at the end of the incubation was consistently higher in the summer- than in the winter-collected cells. The respiratory activity of summer-collected semen was higher, but the fructose uptake and lactate accumulation were lower than in the winter-collected semen. Upon washing, however, the aerobic fructolysis of the summer-collected cells, whether suspended in fructose diluent or in diluted seminal plasma of the same ejaculate, was greater, though the differences were not statistically significant. An exception was the significantly ($P < 0.01$) lower lactate accumulation of twice-washed summer-collected cells suspended in seminal plasma of the same ejaculate. It was interesting to note that summer-collected semen contained a slightly higher fructose content than winter-collected semen, a mean of 7.90 and 5.14 mg/ml of semen, respectively. However, the difference was not statistically significant when the "t" test was applied.

TABLE 5

Comparison of diluent and seminal plasma on the aerobic metabolic behavior of washed bovine spermatozoa

Treatment	Diluent	Mean cell count $\times 10^6$	Motility				Aerobic activity			$+Z_{lactate}$
			Initial		Final		$-Zo_2$	$-Z_{fructose}$	$+Z_{lactate}$	$-Z_{fructose}$
			%	Rate	%	Rate	μl.	μg.	μg.	%
Winter cells										
Winter plasma	NaCl	251.2	48.4	2.8	42.6	2.4	9.6	27.8	19.4	70
	HPO_4	241.8	42.5	2.4	26.1	1.2	5.6	23.5	41.6	177
Summer plasma [a]	NaCl	243.8	49.4	2.6	37.5	2.1	9.9	25.0	10.0	40
(From previous season)	HPO_4	249.2	40.0	2.2	22.2	0.8	5.3	23.0	27.9	121
Summer cells										
Winter plasma	NaCl	359.9	52.8	3.1	48.3	2.9	9.2	36.0	22.4	62
(From previous season)	HPO_4	344.4	50.0	3.0	35.6	1.8	5.6	43.0	37.4	87
Summer plasma	NaCl	359.9	52.8	3.1	50.4	3.0	9.9	28.8	16.6	58
	HPO_4	344.4	50.0	3.0	40.0	2.3	5.6	32.2	31.4	98

[a] One season.

TABLE 6
Summary of the analysis of variance of twice-washed cells resuspended in plasma

	Oxygen uptake	Fructose utilization	Lactic Acid accumulation
Bulls	7.79**	0.90	2.58*
Treatments	11.08**	2.56**	4.09**
W C W P vs. W C S P	0	0.10	0.06
S C W P vs. S C S P	0.59	4.24*	2.71
S C vs. W C	0	8.30**	5.74*

W = winter; S = summer; C = cells; P = plasma.
* = significant ($P < 0.05$).
** = highly significant ($P < 0.01$).

Comparing the responses of twice-washed spermatozoa suspended in diluted plasma (plasma from the same ejaculate and plasma collected during the preceding season) to fructose-containing diluent, the motility at the end of the incubation period of washed cells resuspended in plasma was better than in the diluent containing fructose. The metabolic responses, respiration, fructose uptake, and lactic acid accumulation of these cells were also greater ($P < 0.01$). Twice-washed spermatozoa, whether collected in winter or summer, did not utilize as much substrate as semen. Apparently, subjecting the cells to the mechanical process of washing was injurious.

In the preliminary report of this study, winter-collected cells were recorded to utilize more fructose (8). On re-examination of the values, an error in calculation was noted. Corrected values are as follows: the fructolytic activity ($-Z$ fructose) of winter-collected semen diluted in saline and phosphate was 40.4 and 44.5; of $+Z$ lactate was 18.8 and 27.7, respectively. The $-Z$ fructose of winter-collected, twice-washed cells suspended in winter plasma (diluted plasma from the same ejaculate) diluted with saline and phosphate was 30.0 and 27.0, and in summer plasma (diluted plasma from the preceding season), 25.3 and 20.5. The $+Z$ lactate values for these treatments were 20.2, 40.1, 12.9, and 27.5, respectively. These corrected values are consistent with the final analysis, in that the uptake of fructose by summer- rather than winter-collected, twice-washed cells was greater.

In an experiment of this nature, the confounding of advancing age of the bulls and the passage of the four collection periods, two winters and two summers, over a period of 2 yr. could not be eliminated as a possible source of variance in the metabolic measures. However, comparisons of the metabolic responses of the cells collected in one season of the year with those collected in the same season of the second year indicated that the differences were not marked. Furthermore, it would be difficult to assign the cause of the metabolic differences, had they occurred, to the aging of the bull or to the differences in the climatic conditions of two different years. Since the observed metabolic responses of spermatozoa collected in the same seasons a year apart were not markedly different, it would indicate that the aging effect of the bulls played little, if any, part in the metabolic responses observed between summer- and winter-collected spermatozoa.

These experiments leave little hope that any considerable part of the fertility differences in cattle noted from season to season are associated with changes in the metabolic capacities of the spermatozoa tested immediately after ejaculation. While the relation of fertility and metabolic rate was not directly tested in these studies, the metabolic changes noted as due to seasons were small and required detailed experimental control to demonstrate. Thus, other factors influencing the metabolic rate of spermatozoa would overwhelm the minor seasonal effects in random assays from many bulls. What is even more pertinent is that, in general, the summer-collected cells lived better during the 4-hr. incubation; whereas, in the central part of Illinois the fertility level of cattle tends to be depressed during the hot summer months.

The cause or causes of the metabolic differences of spermatozoa encountered with the seasons of the year are not presently known, and further work is necessary to identify them.

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FAT DIGESTIBILITY BY DAIRY CALVES[1]

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SUMMARY

The utilization of fat in milk replacers by the dairy calf was studied. When a milk replacer composed predominantly of dried skimmilk was supplemented with tallow, coconut fat, grease, or butter, in the unhomogenized state, the fat was poorly digested. However, the inclusion of crude soybean lecithin in the milk replacer improved the utilization of tallow, coconut fat, and grease. The digestibility of coconut fat was improved to a greater extent than was that of tallow or grease by the inclusion of lecithin in the milk replacer.

Milk replacers containing homogenized tallow, lard, butter oil, or hydrogenated soybean oil have been shown to be equal, or nearly equal, to whole milk in promoting growth in the dairy calf. On the other hand, the inclusion of non-hydrogenated vegetable oils, such as soybean oil and cottonseed oil, have been shown to be detrimental to the health of calves (2, 7, 9, 10, 13, 14). The digestibility of certain fats by the dairy calf has been studied to a limited extent. Parrish *et al.* (16) and Noller *et al.* (15) found the apparent digestibility of the ether extract of whole milk to be between 96 and 98%. Blaxter and Wood (3) concluded that the ether extract of whole milk had an apparent digestibility of 96%. A recent report by Raven and Robinson (17) demonstrated that the digestibility of butterfat in spray-dried whole milk was 97% by 2-wk.-old calves. When liquid skimmilk containing homogenized palm oil was spray-dried, the digestibility of the fat was 92%. Wing (20) found that homogenized hydrogenated soybean oil was 92% digested by the dairy calf. Cunningham and Loosli (5) fed calves purified milk replacer diets containing either lard or hydrogenated coconut oil in the homogenized state. They found that coconut fat was 86.4, 89.7, 85.5, 88.6, and 71.9% digested during the second, fourth, sixth, eighth, and 11th wk. of age, respectively. Lard was found to be 72.6, 77.0, 92.5, and 93.7% digested during the second, fourth, sixth, and 11th wk., respectively. Blaxter and Wood (4) noted that lard and a small amount of other fatty material had an apparent digestion coefficient of 44.9% when fed in a nitrogen-free diet. However, the apparent digestibility of this fat was increased if the calories of the diet supplied by protein were increased. They postulated that fat digestibility plateaued at slightly over 90% when between 15 and 18% of the dietary calories were supplied in the form of protein.

In most studies of high-fat milk replacers, the fats have been homogenized with the diet. Little information is available on the value of different fats when mixed directly with the dried milk replacer and fed in the unhomogenized state. Kastelic *et al.* (11), while developing a purified diet for the calf, noted that a

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mixture of butter and soybean lecithin did not need to be homogenized to produce adequate growth. On the other hand, cottonseed oil had to be thoroughly homogenized with lecithin to be a successful dietary ingredient. Huff *et al.* (8) observed a loss of hair about the tail and inside of the thighs of calves fed unhomogenized hydrogenated cottonseed oil with glycerol monostearate as an emulsifying agent. This characteristic alopecia was not noted if the diet was homogenized with or without glycerol monostearate. Lambert *et al.* (12) noted that when calves were fed a milk replacer containing hydrogenated soybean oil, superior growth resulted if lecithin was included in the diet. The following study was initiated to obtain additional data on the utilization of several fats when fed to dairy calves.

EXPERIMENTAL PROCEDURES

Experiments were designed to study the digestibility of the following unhomogenized fats by the calf: (1) tallow, (2) coconut fat, (3) sweet butter, (4) tallow plus crude soybean lecithin, (5) coconut fat plus crude soybean lecithin, (6) homogenized coconut fat and crude soybean lecithin, (7) choice white grease, and (8) choice white grease plus lecithin. Originally, the design included the study of homogenized tallow plus crude soybean lecithin. Sweet butter was substituted for this fat after one period because of difficulties that occurred while attempting to homogenize the tallow. In addition, the fat clumped badly after homogenization, making representative sampling impossible. The first experiment involving Diets 1-6 was a randomized block design, consisting of five blocks of six calves each obtained at a different time over 12 mo. The second experiment, involving Diets 7 and 8, was of a completely random design and conducted later than the first experiment.

The composition of the experimental milk replacer diets is given in Table 1. The basal mixture had the following composition: spray-dried skimmilk, 100; Aurofac D (5 g. of chlortetracycline per pound), 1.50; vitamin A (20,000 I.U. per gram.)[2], 0.25; irradiated yeast (9,000 U.S.P. units of vitamin D per gram),

[2] Nopcay 20, Type IV, generously supplied by Nopco Chemical Co., Harrison, N. J.

TABLE 1
Composition of the high-fat milk replacers used

Diet No.	1	2	3	4	5	6	7	8
Ingredients:	(lb.)							
Basal mixture	80	80	80	80	80	80	80	80
Edible coconut fat[a]		20			18	18		
Bleachable fancy tallow[b]	20			18				
Sweet butter[c]			25					
Choice white grease[b]							20	18
Crude soybean lecithin[d]				2	2	2		2

[a] Stabilized with 10 g. B.H.T. per 100 lb.
[b] Stabilized with Tenox 2 (propylene glycol, B.H.A., and citric acid).
[c] Stabilized with 45 g. B.H.T. per 100 lb.
[d] Alcolec S—American Lecithin Co., Woodside, N. Y.

0.017. In earlier studies at this laboratory, this basal mixture has been observed to be 94% digested by 2-wk.-old calves when fed as a reconstituted milk replacer. The fats used in these studies had the following titers (solidification temperature of the saponified fatty acids; A.O.A.C. *Official Methods of Analysis,* 8th ed.): tallow, 42° C.; coconut fat, 23° C.; butter, 37° C.; grease, 39° C.

The fat that was to be added to the basal mixture was melted and lecithin, when it was included, was added to the melted fat. The melted blend was combined with the basal mixture and stored at barn temperature. At the time of feeding, these diets were reconstituted with water at a ratio of one to ten. The homogenate of Diet 6 was prepared in a Gaulin two-stage homogenizer with 500 and 2,000 lb. p.s.i. pressure at the first and second valves, respectively. The homogenate contained three parts water and one part fat. The particle size of the homogenate was about 2 γ. At the time of feeding, the basal mixture of Diet 6 was reconstituted with water and an appropriate amount of homogenate added. The diets for the second experiment were prepared in a similar manner.

The animals used in these experiments were male Holstein calves obtained from local dairymen or auctions and were approximately three days of age upon arrival at our laboratory. The animals received a diet of whole milk alone for several days after arrival. The milk replacers were gradually substituted for the whole milk, so that after seven days the calves received only 200 g. of the milk replacer, reconstituted at the rate of one part to ten parts of warm water and fed in open pails. No hay or grain was fed. Water and iodized salt were offered ad libitum. The calves were housed in a heated barn in elevated box-type pens with metal-grid floors. In pens such as these, the calves could move about freely and the metal-grid afforded satisfactory footing. Previous attempts to confine calves in stanchions had proved to be unsatisfactory.

Between the ages of 2 and 3 wk., digestibility trials of at least six days' duration were initiated. Fecal collections were made by attaching polyethylene bags to the calves in the manner described by Noller *et al.* (15). In the event of diarrhea during the preliminary period of the digestion trial, the trial was postponed. If diarrhea occurred during the digestion trial the data were, with one exception, discarded. The exception was made in Period Two of the tallow treatment in which the feces were collected for three days, discarded for two days because of diarrhea, and again collected for three days. During the preliminary period of the digestion trials, a carmine marker was fed with the night feeding. The time from the feeding of the marker until its initial appearance in the feces was recorded as transit time.

Feeds were analyzed for fat by extraction in a Soxhlet apparatus. Aliquots of the fat homogenates were analyzed by adding subsamples to tared extraction flasks filled with shredded asbestos. The flasks were dried, and the unevaporated residue of the subsample was calculated as total fat. Samples of dried feces were extracted for 48 hr. with ethyl ether in a Soxhlet apparatus. Following this preliminary extraction, the sample was ground and analyzed for total fat including soaps, by an adaptation of the Saxon method (18). The sum of the extractions was used in calculating the digestion coefficients.

Statistical analyses were made of the digestibility data as follows: An arcsin transformation was made of the digestion coefficients to correct for heterogenous variance as well as non-normality. Missing plots were estimated and an analysis of variance was made by the methods outlined by Snedecor. The upward bias of the treatment mean square caused by the calculation of missing plots was corrected (19). If a significant F value was obtained, the multiple range test of Duncan (6) was used to test the significance of individual means. Except for the transformation, the transit time data were analyzed in a similar manner.

RESULTS AND DISCUSSION

The apparent digestion coefficients of total fat of the experimental diets are given in Table 2. As has been mentioned, the butter treatment was not assigned in the first block. The other missing observations resulted from removal of the animals from experiment, due to scours. Statistical analysis of Experiment I showed the fat digestibility to be significantly higher at the 1% level for the animals fed the diets containing homogenized coconut fat plus crude soybean lecithin and coconut fat plus crude soybean lecithin, than for the animals fed any of the other experimental diets. Tallow plus lecithin was better digested than tallow. In Experiment II (Table 2), the calves fed grease plus lecithin digested the fat significantly better (1% level) than those fed grease alone. No other statistically significant difference was noted.

Apparently, when skimmilk is supplemented with an unhomogenized fat without an emulsifying agent, the fat is rather poorly utilized. However, the inclusion of lecithin in a high-fat diet appears to increase the digestibility of the fat. Because of the excellent utilization of coconut fat plus lecithin in this experiment, homogenization could not be expected to improve its utilization by any appreciable amount. Rather inexact data were collected in the one digestion trial in which the basal mixture was supplemented with homogenized tallow plus lecithin. Due to a sampling problem it was calculated from chemical analyses that between 370

TABLE 2

Apparent digestibility of fat and transit time of diets by dairy calves

Diet	Block 1	2	3	4	5	Av.	Transit time
Experiment I							*(hr.)*
1. Tallow	64.6	52.4		53.8	66.2	59.2	22
2. Coconut fat	66.0	60.1		64.4	71.3	65.4	25
3. Butter		73.3	52.8	66.1	76.9	67.3	22
4. Tallow + lecithin	85.0	68.9	57.1	77.5		72.1	17
5. Coconut fat + lecithin	96.0	97.9	90.4	98.2	95.4	95.6	21
6. Coconut fat + lecithin (homogenized)	90.4	96.1	96.9	96.0		94.9	22
Experiment II							
7. Choice white grease	59.1	44.2	51.1			51.5	
8. Choice white grease + lecithin	77.8	81.2	79.2			79.4	

and 550 g. of fat were fed. However, since only 25 g. of fecal fat were collected, the digestibility of the tallow homogenate must have been between 93 and 96%. This single observation is indicative that homogenization does indeed improve fat utilization of a poorly digested fat and helps explain the good growth responses that have been obtained when homogenized tallow is fed to calves.

The low digestion coefficients obtained from butter are of interest in view of the high digestion coefficients that have been reported for the ether extract of liquid and of spray-dried whole milk. These observations further suggest that the physical form of fat is important for maximum utilization by the calf. It should be noted, however, that significant amounts of the lecithins of whole milk are not present either in churned butter or in skimmilk, but are concentrated in the buttermilk.

It is apparent that crude soybean lecithin improves the digestion of fats by young dairy calves. While this improvement would seem to be due to the action of the lecithin as an emulsifying agent, it may result from some other action or a constituent of the crude lecithin. It is of interest to note that the digestion of the coconut fat, a low-melting fat, was improved more than was the digestion of tallow, a high-melting fat. Auger *et al.* (1) observed that the digestibility of high-melting fats by rats was improved more than that of low-melting fats by the inclusion of lecithin in the diet. However, these workers fed fats with higher melting points than we used, and they observed sizable differences in digestibility between the different fats fed without lecithin.

The transit times of the diet that were obtained with the carmine marker are presented in Table 2. Transit times of the rations were found to vary widely from calf to calf. Analysis of variance of the data gave a nonsignificant F of 1.3. Thus, the speed of passage did not seem to be an important factor in fat digestion in this experiment.

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CHANGES IN THYROID I^{131} RELEASE RATE DURING GROWTH IN HOLSTEIN, BROWN SWISS, AND JERSEY CALVES AT CONSTANT ENVIRONMENTAL TEMPERATURES OF 50 AND 80° F.[1, 2]

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SUMMARY

Studies on the effect of age and temperature on thyroid I^{131} release rate on growing calves were conducted under the controlled environmental conditions of the Missouri Climatic Laboratory.

At both temperatures and for all calves, thyroid I^{131} activity increased with age, whereas thyroid I^{131} activity per unit weight decreased with age. Although all calves showed the same trends, breed differences in the latter relationship were highly significant, Jerseys having the highest ratios, followed by Brown Swiss and Holsteins. The thyroid I^{131} release rate of Jersey calves was approximately twice as great as the rate for Holstein and Brown Swiss, throughout this growth study; however, only the breed-age interaction was significant.

Thyroid activity per unit weight was higher at 80° F. than at 50° F., with highly significant breed differences occurring, and varied significantly as a function of the interaction between temperature and age. Thyroid I^{131} activity per unit surface area showed no effect of temperature alone, but did increase slightly with increments in both age and temperature. Breed differences were significant in the ratio of thyroid I^{131} activity to surface area.

The exponents (b values) of the parabolic equation expressing the relationship of thyroid I^{131} activity to body weight proved to be significantly higher at 80 than at 50° F. There were significant breed differences in percentage increase in thyroid I^{131} activity per 1% increase in body weight, the Jerseys' rate being higher than those of the Brown Swiss or Holsteins.

Thyroid I^{131} activity, believed to be an important factor in growth and milk production, was measured on growing dairy calves in the Missouri Climatic Laboratory. The objective was to determine the effects of 50 and 80° F. constant environmental temperature on the changes in thyroid activity during growth (1–12 mo.).

The studies of Johnson *et al.* (8, 9) on rabbits showed that relatively high constant environmental temperatures depressed thyroid I^{131} release rate. Blincoe (1) demonstrated a similar effect upon beef cattle and Brown-Grant (3) provided evidence that cold environmental temperatures increased the thyroid I^{131} release rate of rats.

EXPERIMENTAL CONDITIONS AND METHODS

Experimental conditions. These studies were conducted under the controlled environmental conditions of the Missouri Climatic Laboratory. The laboratory consists of two independently controlled chambers; one chamber was maintained

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[2] Aided in part by a grant from the U. S. Atomic Energy Commission.

at a constant temperature of 50° F. and the other at a constant temperature of 80° F. Humidity, air velocity, and illumination remained constant.

Holstein, Brown Swiss, and Jersey heifer calves were used in these studies. Three calves of each breed were placed in pens in each chamber at about 2 wk. to 1 mo. of age and were maintained there until the end of the experiment (about 12 mo. of age). Feed composition and management were the same for each chamber.

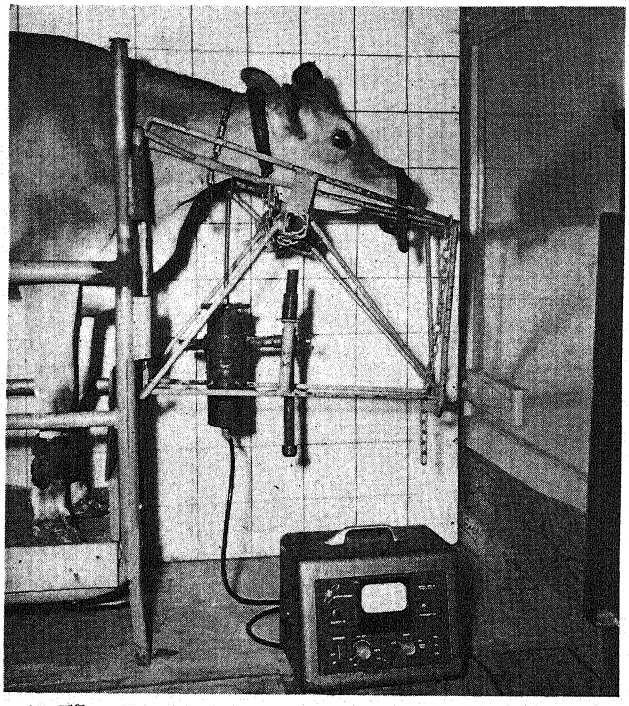


FIG. 1. Thyroid I^{131} activity counting apparatus. The stall is portable; the wheels are removed during the counting procedure.

Calves were fed alfalfa hay ad libitum and grain according to a program similar to that suggested by Herman (5).

Experimental procedures. Approximately every 6 wk. the calves were injected intravenously with carrier-free Na I^{131}. The dose level was 100 μc. until the calves were 6 mo. old, then 200 μc. throughout the remainder of the experiment.

Figure 1 indicates the method for determination of the thyroid I^{131} release rate. The restraining stall and head-holder is portable and the wheels are removed during the counting procedures. The counting stall was moved into the temperature chambers and counts were made daily on the animals, a procedure to which the animals readily became accustomed. An NRD scintillation detector (1 in. crystal) with a 30-cm. spacer was used for the counting procedures. Maximum counts per minute were recorded by a Nuclear Chicago rate meter. With this counting procedure the standard activity was determined (a known amount,

actually one-half of the dose given the animal) at a distance approximately equal to that of the thyroid from the detector. Counts as indicated were made of the thyroid region and, after correction for the standard dilution factor and room background level, the data were expressed as per cent of standard dose. Counts were made at the statistical error level of 0.01. Duplicate readings were taken and the values averaged.

The values were plotted on semilogarithmic paper and the slopes determined from daily data taken from three to approximately 30–35 days after injection of Na I^{131}.[3] The slopes or loss of activity in the thyroid region were determined by the method of least squares to express the rate of thyroid release of I^{131} activity. This slope, or K, is equal to K_4 as reported by Blincoe and Brody (2).

In addition to thyroid I^{131} activity, many physical and physiological measurements were made on the growing dairy calves. These measurements included: body weight—twice weekly; heart girth and wither height—bimonthly; surface area (surface integrator method), hair density (beta gauge), hair length and color—every 6 wk.; rectal temperature, respiration, and pulse rate, feed and water consumption—daily; heat production, respiratory, surface (Capsule), and total vaporization—weekly; and red-cell volume (Cr^{51}), butanol soluble I^{131}, and blood glutathione (GSH)—every 6 wk.

DATA AND DISCUSSION

Changes in thyroid I^{131} activity versus age. The thyroid I^{131} release rate of the three breeds differed, as shown in Figure 2 and in Table 1.

TABLE 1

Analysis of variance of thyroid I^{131} activity

Source of variation	Df	Mean square	F	P
Temperature	1	8.0716	.3750	n.s.
Age	6	36.8352	43.5281	s(.01)
Breed	2	75.4008	3.5029	n.s.
Temperature × age	6	3.7130	4.3876	s(.01)
Temperature × breed	2	13.2191	.6141	n.s.
Age × breed	12	3.0831	3.6433	s(.01)
Temperature × age × breed	12	0.5141	.6075	n.s.
Animal in TB	12	21.5250	25.4361	s
Age × animal in TB	72	0.8462	.....	.....

An analysis of variance, performed according to the outline recommended by Schultz (11), demonstrated that the effect of age upon thyroid I^{131} activity was highly significant. Breed and temperature differences in thyroid I^{131} activity per se were not significant. Although the interaction between temperature and breed was insignificant as well, the interactions between age and temperature and between age and breed were significant.

Thyroid I^{131} activity increased more with age at 80 than at 50° F.; nevertheless, the early data points probably influenced this trend. The early data

[3] Reutilization correction factors were not used, since accurate values for calves of various ages and environmental temperature conditions were not available.

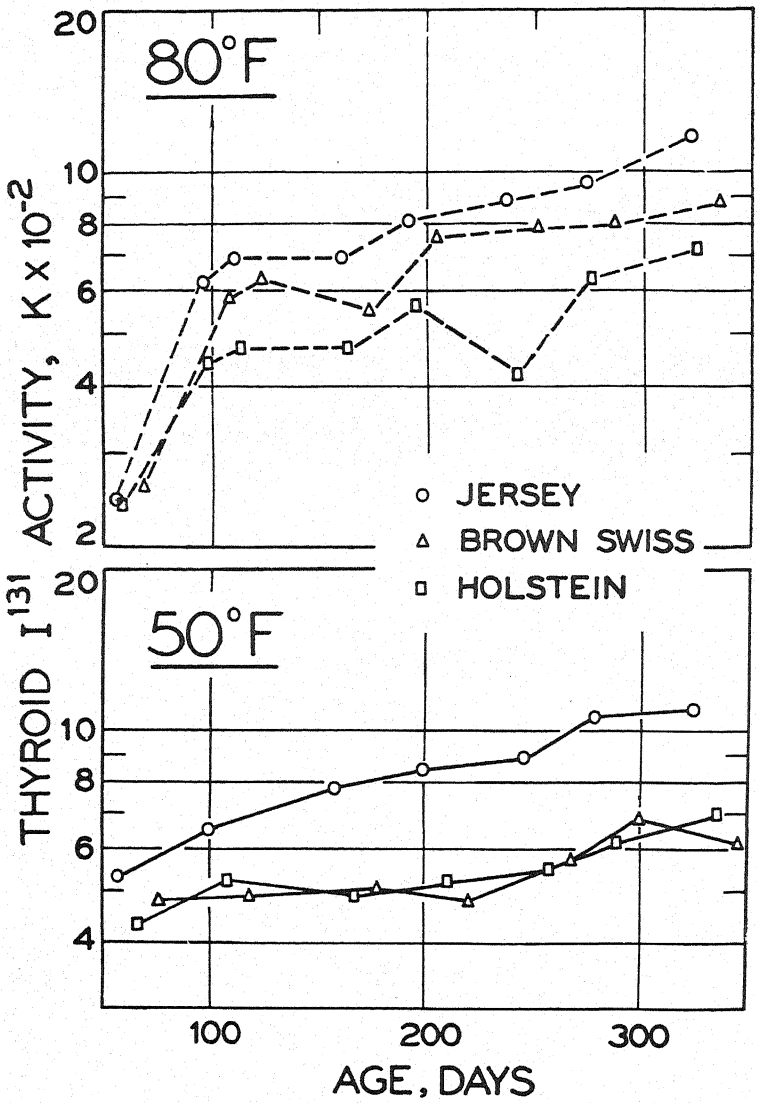


FIG. 2. Thyroid I^{131} activity of Jersey, Brown Swiss, and Holstein calves during growth at 50 and 80° F.

points for the calves in the 50° F. room may be high, because of the fact that the animals were experiencing a considerable constant cold exposure for their relatively small body size. The initially high thyroid I^{131} activity may be a reflection of their extra heat requirements due to greater surface area per unit weight. It is also probable that the animals in the 80° F. chamber were more heat-stressed during the early growth period and, consequently, depressed in their thyroid activity. However, observations of short-period exposures, made by Johnson (7) at the conclusion of the growth experiment, indicated significant 80–50° F. differences. The present similarity in thyroid I^{131} activity is believed due to

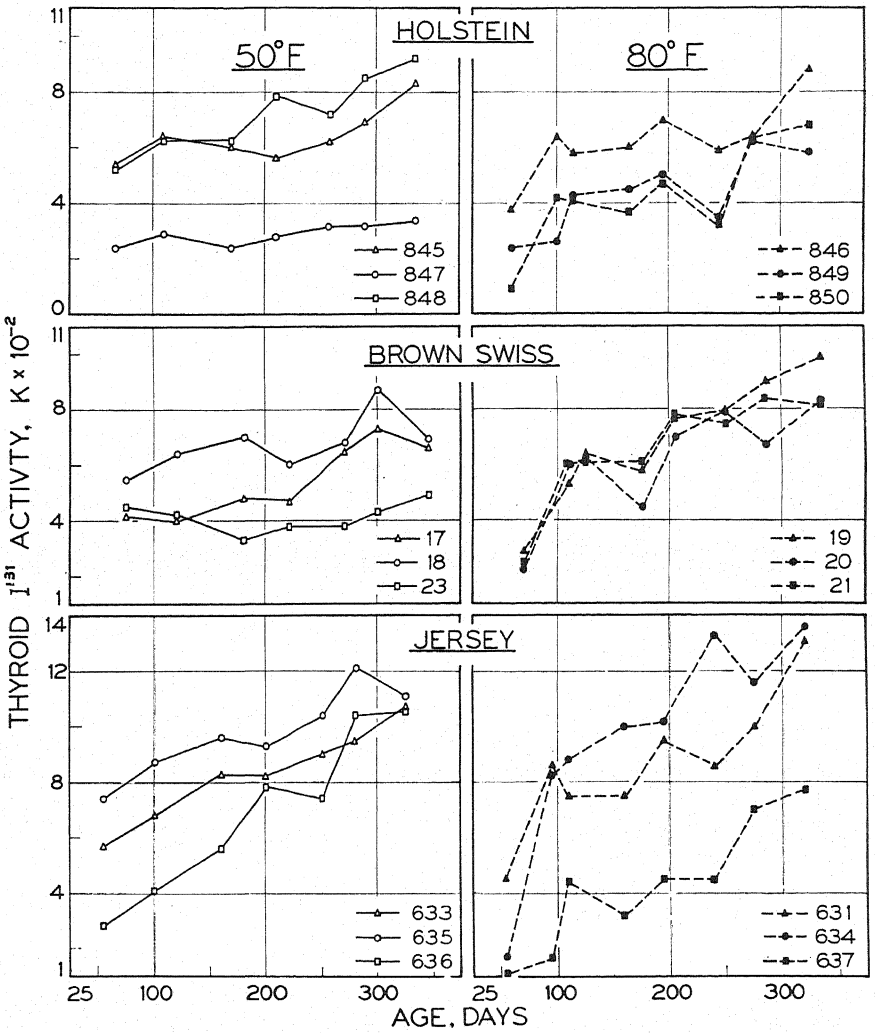


FIG. 3. Thyroid I^{131} activity for individual Holstein, Brown Swiss, and Jersey calves during growth at 50 and 80° F. Graphs indicate considerable individual differences in some breeds, which were constant throughout the growth study.

the relatively much longer period of acclimation, or adaptation of the growing animal to the environment. The exact mechanisms of acclimation, or adaptation, in cattle are unknown at the present time.

Hoesslin (6) observed a similar adaptation phenomenon from results of studies on two-litter mate dogs—one raised at 32° C. and the other at 5° C. Even though there was an enormous temperature difference between the body and the air, the 5° C.–reared dog's metabolism was only 12% higher. However, the cold-reared dog's fur was three times heavier than that of the 32° C. animal. The dairy calves in this experiment appeared to have compensated by this as well

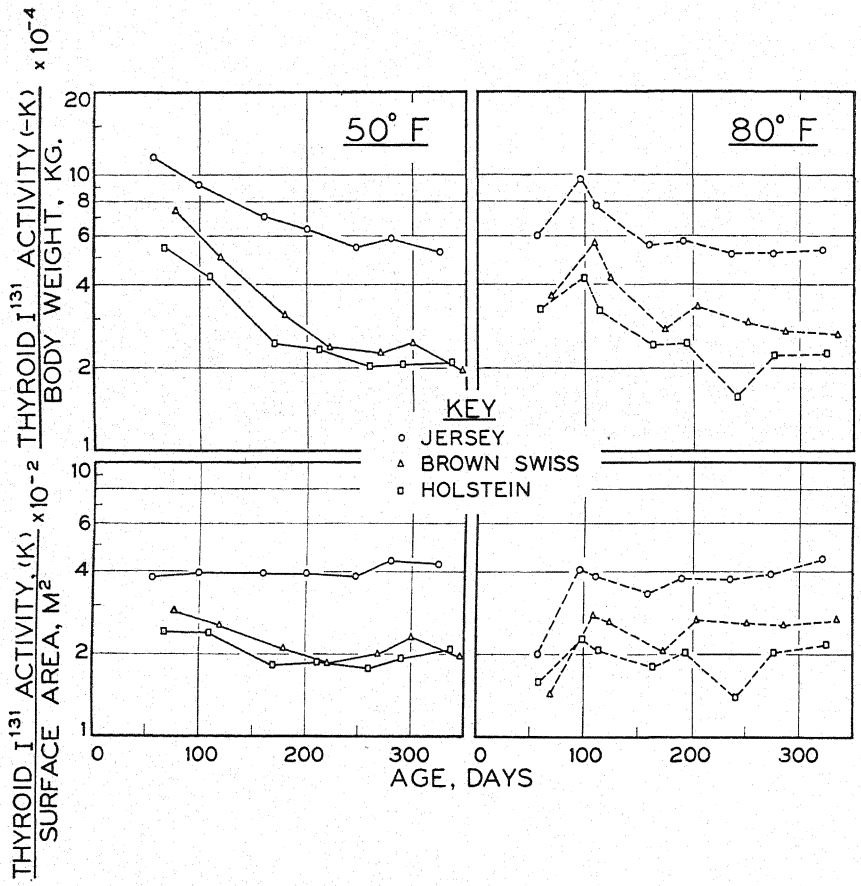


FIG. 4. Thyroid I^{131} activity per unit surface area and per unit body weight for Jersey, Brown Swiss, and Holstein calves raised at 50 and 80° F.

as by other physical and physiological factors as indicated by unpublished hair density data on these calves. For example, the average hair densities for the 50 and 80° F. dairy calves were 12.3 mg/cm and 9.2 mg/cm, respectively.

The influence of the hair coat on mammals is further emphasized by the work of Freinkel and Lewis (4), in which the thyroid activity was reported higher in shorn sheep than in unshorn sheep at the same cold environmental temperature.

Individual animal differences in thyroid I^{131} activity. Striking individual differences were apparent in the 50° F. Holsteins and the 80° F. Jerseys, as indicated graphically in Figure 3. Note the lower level of thyroid activity of Holstein 847 and Jersey 637. These within-breed differences were consistent throughout the growth study. The relationship of these individual differences to heat tolerance or level of production (growth) will be reported later.

Thyroid activity per unit weight. In the ratio of thyroid activity to unit weight (upper section of Figure 4 and Table 2), the Jerseys again had the highest values, the Brown Swiss, intermediate, and the Holsteins, the lowest values per

unit weight throughout the study of growth. These breed differences were statistically significant at the 0.01 level of probability.

TABLE 2

Analysis of thyroid I^{131} activity per unit weight

Source ofvariation	Df	Mean square	F	P
Temperature	1	8.8956	1.0193	n.s.
Age	6	33.4009	29.2117	s(.01)
Breed	2	181.4478	20.7915	s(.01)
Temperature × age	6	10.0190	8.7624	s(.01)
Temperature × breed	2	2.9921	.3429	n.s.
Age × breeds	12	.7986	.6984	n.s.
Temp. × age × breeds	12	.6916	.6049	n.s.
Animal in TB	12	8.7270	7.6325	s
Age × animal in TB	72	1.1434	.....	.....

In younger calves (1–3 mo.), it is generally known that all metabolic functions are the highest per unit weight. At this age the thyroid I^{131} activity is approximately twofold greater than for animals approximately 1 yr. old. This definite trend for thyroid activity per unit weight to decrease with increasing age is evident at both the 50 and 80° F. temperatures. The difference in this ratio as a function of temperature was not significant, whereas the difference as a function of the interaction of age and temperature was again highly significant, with a probability of 0.01.

Relationship of thyroid activity to surface area (M^2). A constant relationship of thyroid activity to growth is shown in the lower section of Figure 4. This graph relates a productive or compensatory productive mechanism such as thyroid activity to the major heat conservation or dissipating surface of the animal. Again, as presented in Table 3, the ratios for the three breeds differ much in the same order, and they are significantly different at the 0.01 level of probability.

TABLE 3

Analysis of thyroid I^{131} activity per unit surface area

Source of variation	Df	Mean square	F	P
Temperature	1	20.9649	.0462	n.s.
Age	6	47.6608	1.0478	n.s.
Breed	2	4551.0574	10.0310	s(.01)
Temperature × age	6	234.6041	6.9295	s(.01)
Temperature × breed	2	152.1525	.3354	n.s.
Age × breed	12	9.1173	.2693	n.s.
Temperature × age × breed	12	33.2174	.9811	n.s.
Animal in TB	12	453.7005	13.4010	s
Age × animal in TB	72	33.8558	.....	.....

Figure 5 and surface area data, Kennedy *et al.* (10), offer some explanation for this relationship. Kennedy demonstrated that the logarithmic relationship of thyroid activity to body weight which varies with the breed and temperature is similar to the logarithmic relationship of surface area to body weight. Although the ratio of thyroid I^{131} activity per surface area does not vary appreciably

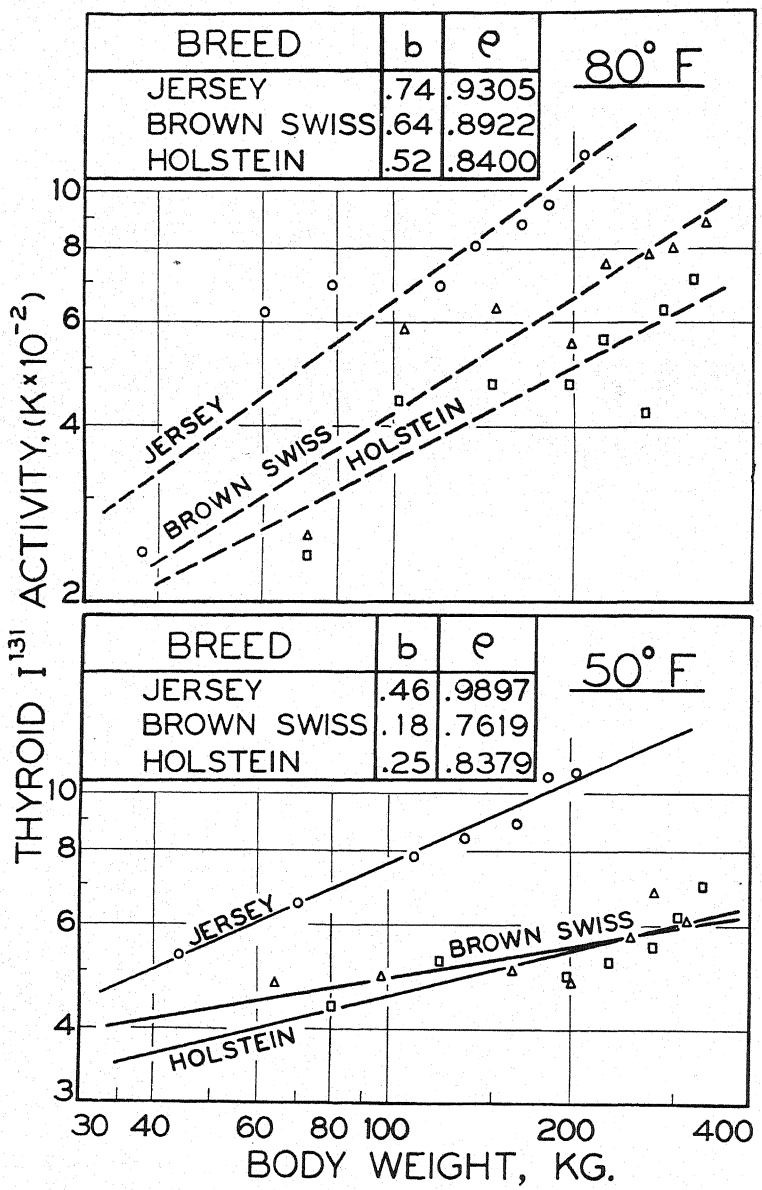


FIG. 5. Thyroid I^{131} activity versus body weight. Levels of significance of the correlation coefficients r were as follows: 50° F.—Jersey 0.01, Brown Swiss 0.05, and Holstein 0.05; 80° F.—Jersey 0.01, Brown Swiss 0.05, and Holstein 0.01.

either with age or with temperature, it does vary significantly, at the 0.01 level, as a function of the interaction between age and temperature.

In interpreting these ratios (thyroid I^{131} activity per unit weight and per unit surface area), the magnitude of the denominators must be considered. At both temperatures, the body weights of the Jerseys were considerably lower than

those of the Holsteins and Brown Swiss; the surface area values of the Jerseys also were considerably lower. The body weights and surface area values of the Brown Swiss and Holsteins were fairly similar to each other.

Considerable evidence indicates that thyroid activity is determined by the tissue demand for the thyroid hormone (12). Since the thyroid function, like metabolism, represents a generalized activity, it appears to be justifiable to express the data in the given ratios.

The logarithmic relationship of thyroid I^{131} activity to body weight. The regression lines in Figure 5, obtained from averages of three individuals in each breed at each temperature, are expressed by the equation $Y = aX^b$, with the thyroid activity as the dependent variable. An increase in weight (X) by 100% is associated with an approximate differential increase in Y (thyroid activity) by as low as 0.18 to as high as 0.74, respectively, for the 50° F. Brown Swiss and 80° F. Jerseys. Mean exponents for the 80° F. Jerseys were: 0.74 as compared to 0.46 for the 50° F. Jerseys; Brown Swiss, 0.64, at 80° F. as compared to 0.18 at 50° F.; and 0.52 for Holsteins at 80° F. and 0.25 for the 50° F. group.

Table 4 shows a statistical comparison of the individual b values.

The relationship of thyroid activity to body weight was significantly different (.05 level of probability) at the two temperatures. Breed differences also were highly significant (P = .01).

TABLE 4

Analysis of variance of "b" values from thyroid I^{131} activity vs. body weight (kg.)

Source of variation	Df	Mean square	F	P
Temperature	1	.00032496	5.9978	s(.05)
Breed	2	.00134168	24.7634	s(.01)
Temperature × breed	2	.00001645	.3036	n.s.
Animal in TB	12	.00005418	.....	.....

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OBSERVATIONS ON THE NUTRITION OF *BACTEROIDES SUCCINOGENES*—A RUMINAL CELLULOLYTIC BACTERIUM

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SUMMARY

Studies on five strains of *Bacteroides succinogenes,* including one recent isolate, indicated that biotin was essential for growth of four strains and highly stimulatory for the other. *p*-Aminobenzoic acid stimulated the growth of two strains. No other vitamins had an effect on growth. Ammonia was essential for growth even in the presence of 19 amino acids, a mixture of purines and pyrimidines, and all B vitamins. None of the amino acids were essential for growth, and good, but somewhat delayed, growth was obtained when cysteine or glutathione was the only source of amino acids available. Studies on Strain S85 indicated that cysteine or sulfide could serve as the sole source of sulfur. The sulfur requirement was not adequately studied, as the only reducing agents found that allowed good growth in chemically defined media were those that also served as a sulfur source. The essentiality of PO_4^{---}, NH_4^+, Mg^{++}, Ca^{++}, K^+, and Na^+ was demonstrated and the levels required were established for Strain S85. Although glucose was used as an energy source in most experimental media, it was demonstrated that good growth and cellulose digestion occurred in a medium containing only cellulose, *p*-aminobenzoic acid, biotin, cysteine, alanine, phenylalanine, valerate, isobutyrate, a carbonic acid–bicarbonate buffer, resazurin, and minerals. The results indicate the relatively great ability of *B. succinogenes* to synthesize cellular constituents and, also, how well this bacterium is adapted to the environment of the rumen.

It is now generally known that the nutrient requirements of ruminants for efficient utilization of feeds are intimately associated with reactions occurring in the rumen as well as the essential and stimulatory nutrients of ruminal microorganisms. Also, knowledge of the nutrients involved in growth is essential to an understanding of the ecology of an organism. While animal feeding trials and in vitro experiments involving the mixed microbial population have been used to good advantage in advancing knowledge of the nutrition of ruminal organisms, it is evident that studies of pure cultures of judiciously selected species may bring forth information difficult to obtain or difficult to interpret with other methods.

Data of Hungate (20) and others (7, 8, 9) have indicated that *Bacteroides succinogenes* is one of the more important cellulolytic species in the rumen. Previous studies on the nutrient requirements for growth of this organism indicated that anaerobic conditions (9, 20), carbon dioxide, or bicarbonate (9), an energy source such as glucose, cellobiose, or cellulose (9, 20), and a two-component volatile fatty acid mixture (10) are required for growth.

The present work was initiated to obtain knowledge of the B vitamin, nitrogen, sulfur, and mineral requirements of this species.

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METHODS

The strains studied were S61, S85, S121, and M13, many characteristics of which were reported previously (9, 10), and a more recent isolate, CB40, that was isolated in April, 1957, from a steer on a feed-lot, bloat-provoking ration. The latter strain was only presumptively identified as *B. succinogenes* on the basis of morphology, nonmotility, gram reaction, cultural characteristics, relations to oxygen, digestion of cellulose, final pH in glucose medium, and nonproduction of gas and H_2S.

The anaerobic methods used to prepare the media and grow the bacteria were essentially the same as described by Bryant and Doetsch (10), except as indicated below. The composition of the inoculum medium is shown in Table 1.

TABLE 1
Composition of inoculum medium[a]

	(mg/100 ml.)		*(mg/100 ml.)*
KH_2PO_4	90	Glucose	300
NaCl	90	Cysteine · HCl · H_2O	100
$(NH_4)_2SO_4$	90	Casein hydrolysate[b]	200
$CaCl_2$	9	Thiamin · HCl	0.2
$MgSO_4$	9	Pyridoxamine · HCl	0.1
$FeSO_4 \cdot 7\ H_2O$	4	Ca-pantothenate	0.2
$MnSO_4 \cdot H_2O$	0.15	Riboflavin	0.2
$CoCl_2 \cdot 6\ H_2O$	0.15	Nicotinamide	0.2
Na_2CO_3	400	*p*-Aminobenzoic Acid	0.01
Sodium *n*-valerate	7.44	Biotin	0.005
Sodium isobutyrate	3.33	Folic Acid	0.005
Resazurin	0.1	Cobalamin	0.0005

[a] The medium was in equilibrium with CO_2 gas that was passed through a column of hot reduced copper filings to remove traces of O_2.
[b] Nutritional Biochemicals Corporation vitamin-free casein hydrolysate (enzymatic).

The strains were carried with daily transfers of one 4-mm. loop in 5 ml. of inoculum medium 1 in 13 by 100-mm. pyrex culture tubes. At least seven transfers were made before inocula were prepared. They were inoculated at approximately 4 P.M., incubated at 37° C. until 8 A.M., and left at room temperature until inoculum was prepared. Leaving them at 37° C. for longer periods resulted in lysis of cells (10) and erratic growth. Inoculum was prepared by centrifuging down the cells, decanting the medium, and making up the cells to an optical density (O.D.) of 0.1 with anaerobic dilution solution containing 1.2% KH_2PO_4, 1.2% NaCl, 0.1% cysteine · HCl, and 0.4% Na_2CO_3, and equilibrated with CO_2 gas. Sterile solutions of cysteine · HCl and Na_2CO_3 were prepared and added to the solution after autoclaving, as previously described (10). O.D. was determined in calibrated 13- by 100-mm. pyrex culture tubes using a Spectronic 20 colorimeter set at 600 mμ. Except when centrifuging, the cells were maintained under CO_2 in rubber-stoppered tubes. One-tenth milliliter was used to inoculate each 5 ml. of medium. Preliminary experiments indicated Strain S85 required biotin. Using biotin-deficient medium, it was found that washing the cells two or three times resulted in no less carry-over of biotin.

The basal medium for the study of vitamin requirements was the same as the inoculum medium, except that vitamins were deleted. The medium was tubed in

4-ml. amounts, so that addition of 1 ml. of the various vitamin solutions brought it to final concentration. Vitamin mixtures were sterilized for 10 min. at 15 lb. in absorbent cotton-plugged tubes or in tubes with aluminum caps. On removal from the autoclave, the tubes were gassed well with CO_2 and closed with sterile rubber stoppers to prevent entrance of O_2 until ready for addition of the vitamin solutions to the basal medium.

The basal medium for determining nitrogen requirements was similar to the inoculum medium except that L-amino acids were substituted for the casein hydrolysate. Amino acids included were serine, alanine, tryptophane, aspartic acid, arginine · HCl, histidine · HCl · H_2O, lysine · HCl, valine, glycine, proline, hydroxyproline, leucine, isoleucine, threonine, methionine, and phenylalanine at concentrations of 0.01%, and glutamic acid and tyrosine at 0.02% and 0.0017%, respectively. Amino acids were added to the medium before autoclaving. Specific variations in media are given with the results.

In studying the mineral requirements of Strain S85, a number of changes were made in the experimental procedure. Cells used as inoculum were grown in the same manner as above, except that a clarified rumen fluid (CRF) glucose medium was used. This medium was of the following composition: 20% CRF, 0.0005% indigo carmine, and 0.09% $(NH_4)_2SO_4$. Cysteine, glucose, carbon dioxide, and sodium carbonate were added as in the original inoculum medium (Table 1). The CRF was prepared from freshly collected rumen fluid from a cow on an alfalfa hay–grain ration. It was filtered through gauze, allowed to stand until particles rose to the surface or sedimented and the clearer liquid was siphoned off. This was centrifuged at approximately 14,000 r.p.m. for 20 min. and the clear supernatant was sterilized under CO_2 by autoclave (15 lb/20 min.) and refrigerated until used.

The basal medium in which minerals were varied contained glucose, indigo carmine, Na_2CO_3, and cysteine as in the CRF medium. Other ingredients were as follows in milligrams per 100 ml.: sodium isovalerate, 3.72: sodium valerate, 7.44; *p*-aminobenzoic acid, 0.01; biotin, 0.005; and alanine, isoleucine, serine, phenylalanine, tryptophane, glutamic acid, lysine, arginine, and proline, ten of each. The media were adjusted to pH 6.5 with NaOH, H_2SO_4, or HCl before autoclaving (10 min/15 lb.) and adding Na_2CO_3 and cysteine. After the pH was adjusted, it was held under a gas mixture of 80% CO_2 and 20% H_2. It was tubed in 4-ml. amounts, so that addition of 1 ml. of mineral solution brought the ingredients to final concentration.

Minerals used were of A.C.S. quality and each salt was prepared individually in screw-cap bottles and autoclaved at 15 lb. for 10 min. For preparation of the various mixtures, the appropriate amounts of the individual solutions were mixed in sterile aluminum-capped tubes. They were steamed in an Arnold sterilizer for 10 min. or longer and gassed with the CO_2-H_2 mixture to remove O_2. One-milliliter additions were made to the basal medium.

Water used to double-rinse glassware and in culture media was distilled, passed through a column of Amberlite MB-3 ion exchange resins, distilled from Pyrex glass, and stored in polyethylene bottles.

The washed inoculum was prepared as follows: About 0.8 ml. of 18-24 hr. culture was diluted to 3 ml. with the basal medium, with water added in place of the mineral mixture. The cells were centrifuged down at about 12,000 r.p.m. The pellet of cells was washed with the medium and then resuspended and centrifuged down again. The supernatant was decanted and the cells were resuspended and diluted out to O.D. 0.1. Each 5 ml. of medium was inoculated with 0.1 ml. of the suspension.

The O.D. of growth media with indigo carmine added in place of resazurin was determined, utilizing a wave length of 475 mμ.

RESULTS

Vitamin requirements. Single deletion of vitamins from the medium indicated that only biotin and *p*-aminobenzoic acid (PABA) had an effect on growth. Some of the results are shown in Table 2. Biotin was essential for growth of

TABLE 2

Effect of vitamins on growth of strains of *B. succinogenes*

Vitamins added	Growth (O.D. × 100)				
	Strain S85	Strain M13	Strain S121	Strain S61	Strain CB40
All vitamins[a]	96 (24)[b]	105 (24)	82 (24)	96 (24)	92 (27)
All except biotin	1 (24)	1 (24)	28 (48)	3 (24)	3 (24)
All except PABA	93 (24)	102 (24)	79 (24)	52 (48)	44 (112)
Biotin and PABA	92 (24)	100 (24)	82 (24)	96 (27)	82 (27)
Biotin	96 (24)	105 (24)	80 (24)	17 (27)	7 (24)

[a] Thiamine · HCl, pyridoxamine · HCl, Ca-pantothenate, riboflavin, nicotinamide, PABA, biotin, folic acid, and cobalamin.

[b] The numbers in parentheses refer to the hours of incubation for the maximum O.D. reading.

four strains and quite stimulatory for Strain S121. That biotin was not carried over in the inoculum with Strain S121 was indicated by the fact that growth occurred through five successive daily serial transfers in this medium. PABA was stimulatory to Strains S61 and CB40. It was much more stimulatory when added to the medium containing biotin only than when all of the other vitamins were present. Strains S85, M13, and S121 exhibited maximal growth when biotin was the only vitamin present and Strains S61 and CB40 grew well with only biotin and PABA present.

TABLE 3

Effect of certain vitamins on growth of Strain S121[a]

Vitamins added	O.D. × 100	Vitamins added	O.D. × 100
None	2 (16)	Nicotinamide	57 (116)
All except biotin	62 (88)	Cobalamin	54 (88)
Biotin	92 (24)	Thiamine	49 (116)
PABA	47 (116)	Pyridoxamine	60 (96)
Riboflavin	48 (116)	Folic Acid	42 (88)
Ca-pantothenate	48 (88)		

[a] See footnotes of Table 2.

Data in Table 3 show that no appreciable growth of Strain S121 occurred when all vitamins were deleted. Yet slow and limited growth occurred with each vitamin added singly to the medium, except in the case of biotin, which allowed optimum growth. These observations might suggest that each of the vitamins either partially replaces biotin or that the medium was contaminated with biotin. However, it does not seem logical that all of these vitamins might partially replace biotin. The fact that the control medium with no vitamins added did not allow appreciable growth makes it seem evident that if biotin was a contaminant its source was the other individual vitamins. It likewise does not seem possible that this could occur.

Figure 1 shows the response of Strain S85 to different levels of biotin.

Nitrogen requirements. In preliminary experiments it was shown that purines and pyrimidines previously included in the medium (10) had no appreciable effect on growth. Data in Table 4 indicate that 18 L-amino acids can replace the

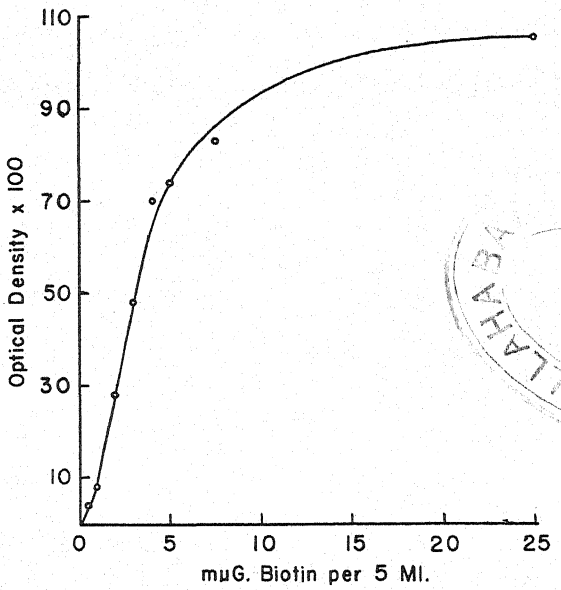


FIG. 1. The response of Strain S85 to varying levels of biotin.

casein hydrolysate in the growth of all five strains and that good, but somewhat delayed, growth is obtained when cysteine or glutathione, added as reducing agent, is the only source of amino acids in the medium. Experiments outlined below indicate that good growth can be obtained with sulfide used in place of cysteine. Thus, no amino acid is essential for growth.

The results indicate that the number of vitamins essential for growth is not increased when all amino acids except cysteine are deleted from the medium. In Experiment 2 of Table 4, PABA and biotin were the only vitamins present.

Data in Experiment 1 of Table 4 indicate that growth did not occur when Na_2SO_4 replaced $(NH_4)_2SO_4$ in the medium. Other experiments showed that

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TABLE 4

Effect of some nitrogen sources on growth of strains of *B. succinogenes*[a]

Ingredients added to basal medium	Growth (O. D. × 100)				
	Strain S85	Strain M13	Strain S121	Strain S61	Strain CB40
Experiment 1[b]					
Casein hydrolysate, $(NH_4)_2SO_4$	100(24)	100(24)	96(24)	96(40)	90(40)
18 L-amino acids, $(NH_4)_2SO_4$	82(24)	96(24)	96(40)	88(40)	92(40)
18 L-amino acids, Na_2SO_4	4(51)	5(24)	3(51)	2(95)	3(68)
$(NH_4)_2SO_4$	77(68)	77(51)	80(68)	80(76)	1(68)
Na_2SO_4	0(119)	0(119)	0(119)	0(119)	0(119)
Experiment 2[c]					
Casein hydrolyysate + cysteine	110(25)	105(18)	89(18)	96(40)	96(25)
Cysteine	100(42)	89(50)	82(50)	105(40)	40(108)
Glutathione (0.1%)	100(42)	82(42)	82(42)	96(40)	60(42)

[a] See Footnote [b], Table 2.

[b] The basal medium was that shown in Table 1, except that casein hydrolysate and $(NH_4)_2SO_4$ were deleted and 0.5 mg. each of adenine, guanine, uracil, xanthine, and thymine per 100 ml. of medium were added.

[c] The basal medium was that shown in Table 1, but minus casein hydrolysate, cysteine, and all vitamins except PABA and biotin.

the Na_2SO_4 was not toxic when included in media in addition to $(NH_4)_2SO_4$. These results indicate that ammonia is essential for growth even in the presence of 19 amino acids, purines, and pyrimidines, and all of the B vitamins.

Using Strain S85, attempts were made to determine the effects of various amino acid mixtures on growth in the medium in Table 1 modified by deletion of casein hydrolysate. Cysteine was always present as reducing agent. Growth was good regardless of the amino acids added, but often a long lag occurred before growth was initiated. In early work it was found that alanine was the only deletion that slowed growth when single deletions were made from mixtures of all 18 amino acids or from a mixture of ten. When various mixtures of five or less amino acids were present, it was found that alanine alone did not support rapid growth, but rapid growth was obtained when alanine plus phenylalanine and/or tryptophane were present. Further experiments indicated that growth was quite slow if both phenylalanine and tryptophane were deleted from the complete mixture, or from mixtures containing ten or less amino acids. Tyrosine would not replace the two other aromatic amino acids. In fact, it was somewhat inhibitory. At this stage, experiments became quite erratic and often could not be reproduced. The study of amino acids was discontinued. The reasons for the erratic results are not known, but it seems probable that, though the resazurin was always reduced when the media were inoculated, they were not always poised at a low enough E_h to allow rapid growth. Different mixtures of amino acids in the simple basal medium of known composition might have a considerable effect on E_h. It is also possible that some of the erratic results were due to amino acid inhibitions and antagonisms.

Excellent and quite reproducible growth was possible in the medium containing ten amino acids. The results of one experiment on the growth obtained in this medium as compared to casein hydrolysate, alanine, phenylalanine, and

cysteine, and cysteine only, is shown in Figure 2. It should be noted that in some experiments growth in the medium containing three amino acids or only cysteine was more comparable to that in media containing casein hydrolysate or ten amino acids.

Sulfur requirements and reducing agents. From the results above it was evident that cysteine and/or sulfate could serve as the sole source of sulfur, other than that present in biotin, for growth of the organism. To obtain more information on the sulfur requirements, attempts were made to replace the cysteine with nonsulfur-containing reducing systems. Strain S85 was used. Table 5 shows some of the results obtained in an attempt to replace cysteine with ascorbic acid. It is evident that ascorbic acid would not replace cysteine as reducing agent, even when levels of cysteine too low to reduce the medium by itself (0.01% or less) were present. The 0.01% level of cysteine undoubtedly would meet the sulfur requirements for maximum or at least near-maximum growth. The fact that good growth occurred when 0.05% of cysteine was present in addition to ascorbic acid indicates that ascorbic acid was not inhibitory to the organism. It seems probable that, though the ascorbic acid alone reduced the resazurin in the medium, the E_h was too high to allow initiation of growth.

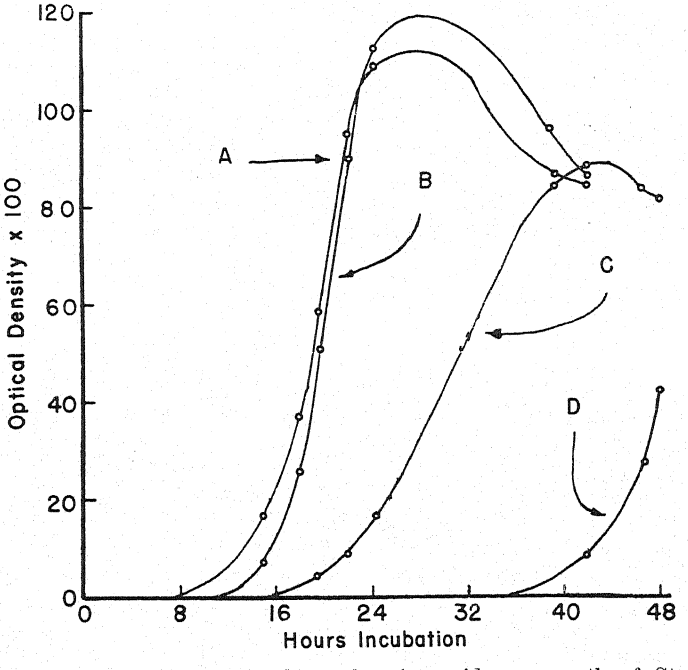


FIG. 2. The effect of different numbers of amino acids on growth of Strain S85. The basal medium was the same as that shown in Table 1, except that casein hydrolysate and all of the B vitamins but PABA and biotin were omitted and the level of cysteine was 50 mg. A, alanine, isoleucine, serine, phenylalanine, tryptophane, glutamic, lysine, arginine, and proline; B, casein hydrolysate; C, alanine and phenylalanine; D, no amino acids other than the cysteine present in the basal medium.

TABLE 5

Effect of some reducing agents and/or sulfur sources on growth of Strain S85[a]

Ingredients added to basal medium[b]	Growth (O. D. × 100)
Experiment 1	
Cysteine · HCl (0.05%)	105 (22)
Ascorbic Acid (0.05 or 0.1%)	0 (72)
Ascorbic Acid (0.05 or 0.1%) + cysteine · HCl (0.05)	89–105 (22)
Ascorbic Acid (0.05 or 0.1%) + cysteine · HCl (0.001, 0.005, or 0.01%)	0 (72)
Experiment 2	
Cysteine · HCl (0.05%)	101 (24)
$Na_2S \cdot 9\ H_2O$ (0.025 or 0.125%)	110 (24)
Experiment 3	
Cysteine · HCl (0.05 or 0.25%)	102–107 (20)
Sodium thioglycolate (0.1 or 0.025%)	0 (112)
Sodium thioglycolate (0.05%)	13 (112)

[a] See Footnote [b], Table 2.

[b] The basal medium was the same as Medium A (Figure 2), except that cysteine · HCl was deleted. The reducing agents, ascorbic acid and thioglycolate, were added both before the basal medium was sterilized and after sterilizing. In the latter case, they were sterilized separately, both by autoclave and by glass filter. Similar results were obtained, regardless of the treatment.

An attempt was made to replace the cysteine-reducing system with palladium chloride or palladium black-hydrogen reducing systems similar to those used by Mylroie and Hungate (27) for isolation of sludge methane bacteria. However, in media containing all known constituents the lowest levels of palladium that allowed reduction of the medium (0.001-0.002%) were toxic to the bacterium.

Results in Table 5 show that sodium thioglycolate allowed little growth but that sodium sulfide was quite satisfactory in replacing cysteine as reducing agent.

Data in Table 6 show that cysteine or $Na_2S \cdot 9\ H_2O$ served quite well as the sole source of sulfur for growth. The amounts required as sulfur source could not be determined, because higher levels were necessary to reduce the medium.

An attempt was made to grow the organism in the basal medium (Table 6) with $(NH_4)_2SO_4$ as the sole source of sulfur and no reducing agent added. A large inoculum, 0.5 ml. of unwashed, undiluted cells (O.D. 1.15), was used in the hope that it would reduce the medium, and daily serial transfers could

TABLE 6

Some data on the effect of cysteine and sulfide as the sole source of sulfur on Strain C85[a]

Ingredients added to basal medium[b]	Growth (O.D. × 100)
Experiment 1	
Cysteine + $(NH_4)_2SO_4$[c]	92 (21)
Cysteine + NH_4Cl	82 (21)
Experiment 2	
Cysteine + $(NH_4)_2SO_4$	96 (34)
Cysteine + NH_4Cl	77 (34)
$Na_2S \cdot 9\ H_2O + NH_4Cl$	70 (34)

[a] See Footnote [b], Table 2.

[b] The basal medium was that used in the study of mineral requirements, except that cysteine, sulfide, and ammonium salts were present as indicated. Minerals added included KH_2PO_4, 0.09%; NaCl, 0.09%; $CaCl_2 \cdot 6\ H_2O$, 0.002%; $MgCl_2 \cdot 6\ H_2O$, 0.0016%; $CoCl_2 \cdot 6\ H_2O$, 0.0004%.

[c] Cysteine · HCl · H_2O, $(NH_4)_2SO_4$, and NH_4Cl were added at the 0.05%, 0.09%, and 0.077% levels, respectively.

be made to dilute out sulfur compounds in the inoculum. However, reduction of the medium and growth occurred only on the first transfer. On addition of 0.1% ascorbic acid, the same results were obtained.

Mineral requirements. It is evident from data in Table 7 that when PO_4^{---}, K^+, Mg^{++}, NH_3, or Ca^{++} was deleted from the otherwise complete medium little or no growth occurred. Fe^{++}, Co^{++}, and Mn^{++} had no effect on growth, indicating that they were not essential or that they were present in the medium as contaminants. About 0.3 mg. of PO_4^{---}, 0.2 to 0.3 mg. of NH_3, 0.01 mg. Mg^{++}, and 0.05 mg. of Ca^{++} per 5 ml. of medium were required for maximum growth (Figures 3 and 4). Whether other cations would spare or replace the Ca^{++}

TABLE 7

Effect of deletion of minerals on growth of Strain S85[a]

Ion deleted	Growth (O.D. × 100)	Ion deleted	Growth (O.D. × 100)
None deleted[b]	93 (41)	Ammonium	0 (97)
Phosphate[c]	0 (97)	Calcium	9 (97)
Potassium[d]	0 (97)	Cobalt	89 (41)
Magnesium	16 (33)	Manganese	85 (41)
Iron	85 (41)		

[a] See Footnote [b], Table 2.

[b] Minerals added to the basal medium included KH_2PO_4, 0.09%; NaCl, 0.09%; $(NH_4)_2SO_4$, 0.09%; $MgSO_4 \cdot 7\ H_2O$, 0.002%; $FeSO_4 \cdot 7\ H_2O$, 0.0005%; $CaCl_2 \cdot 6\ H_2O$, 0.002%; $CoCl_2 \cdot 6\ H_2O$, 0.0004%; and $MnSO_4 \cdot H_2O$, 0.001%.

[c] KCl, 0.056%, was added in place of KH_2PO_4.

[d] $NaH_2PO_4 \cdot H_2O$, 0.09%, was added in place of KH_2PO_4.

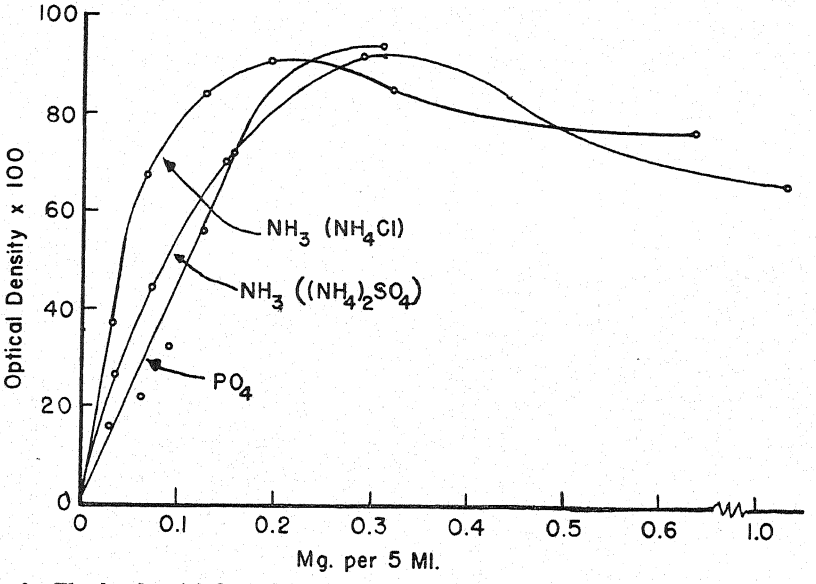


FIG. 3. The levels of PO_4 and NH_3 required for growth of Strain S85. Minerals added to all three media included NaCl, 0.09%; $MgCl_2 \cdot 6\ H_2O$, 0.0016%; $CaCl_2 \cdot 6\ H_2O$, 0.002%, and $CoCl_2 \cdot 6\ H_2O$, 0.0004%. The PO_4-deficient medium contained $(NH_4)_2SO_4$, 0.09%; KCl, 0.056%; and PO_4 was added as $NaH_2PO_4 \cdot H_2O$. The NH_3-deficient media contained KH_2PO_4, 0.09% and, when NH_3 was added as NH_4Cl, 0.09% of Na_2SO_4 was included.

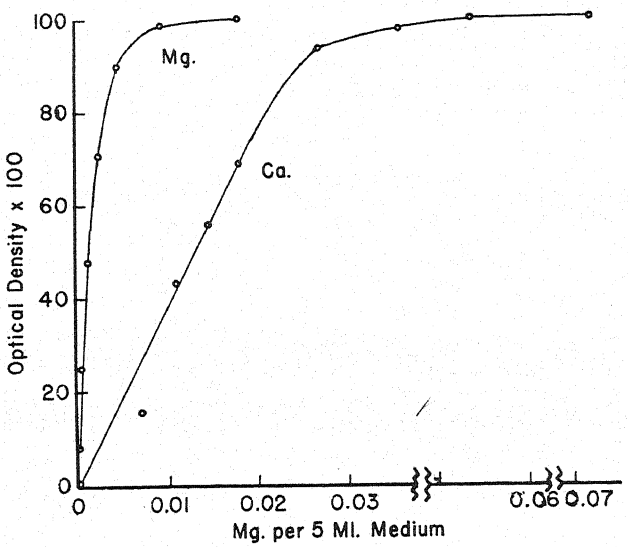


FIG. 4. The levels of magnesium and calcium required for growth of Strain S85. Minerals added to the basal medium included KH_2PO_4, 0.09%; $(NH_4)_2SO_4$, 0.09%; NaCl, 0.09%, and $CoCl_2 \cdot 6\,H_2O$, 0.0004%. $CaCl_2$, 0.001%, and $MgCl_2 \cdot 6\,H_2O$, 0.0016%, were added to the Mg- and Ca-deficient media, respectively, and the same salts were utilized in determining levels required.

or Mg^{++} was not determined. However, it was evident that both Ca^{++} and Mg^{++} were required in the presence of the other and that Fe^{++}, Co^{++}, or Mn^{++} would not replace them in the low levels used.

It was advantageous to include the low level of Co^{++} because the medium reduced at a much slower rate when reducing agent was added without it. Five p.p.m. of Co^{++} inhibited growth and 15 p.p.m. completely stopped growth.

It was evident (Table 8) that K^+ was essential for growth. The 0.082 *N* level of Na^+ was the lowest added to the medium equilibrated with the 80% CO_2 gas, due to the necessity of adding alkali to adjust the pH. At this level of Na^+, best growth occurred at about 0.01 *N* K^+. At the 0.098 *N* level of Na^+, the amount

TABLE 8

Effect of different levels of potassium and sodium on growth of Strain S85 in potassium-free medium[a]

	Growth (O. D. × 100)			
Normality Na^+	0.082	0.098	0.164	0.328
Normality K^+				
0.05	48 (24)[b]	30 (24)	2 (19)	0 (48)
0.01	85 (22)	74 (24)	19 (22)	0 (48)
0.003	64 (22)	92 (24)	24 (24)	0 (48)
0.001	3 (19)	0 (48)	2 (19)	0 (48)
0.0003	0 (48)	0 (48)	0 (48)	0 (48)

[a] Minerals added to the basal medium included $(NH_4)_2SO_4$, 0.09%; $NaH_2PO_4 \cdot H_2O$, 0.09%; $CoCl_2 \cdot 6\,H_2O$, 0.0004%; $CaCl_2$, 0.001%; $MgCl_2 \cdot 6\,H_2O$, 0.0016%, and K was added as KCl.
[b] See Footnote [b], Table 2.

regularly added to media, the best growth occurred with 0.003 N K^+. At higher levels of Na^+, growth was poor, regardless of the K^+ level.

When sodium-free medium equilibrated with 80% CO_2 was prepared with K_2CO_3 and KOH substituting for the sodium compounds, very poor growth occurred at the lowest level of K^+ (0.082 N) and none occurred at the 0.164 N level. At the 0.082 N level of K^+, maximum O.D. was 0, 4, 21, and 0 for 0.3, 0.1, 0.03, and 0.01 N Na^+, respectively.

To study the effect of wider ranges of Na^+ and K^+ concentrations, the CO_2 in the gaseous phase of the basal medium was lowered by using a gas mixture containing 10% CO_2 and 90% N_2, and the Na_2CO_3 concentration was lowered to 0.12%. Growth in this basal medium was rather poor and erratic, probably because of the high bicarbonate requirement of the organism (9) and the lowered buffer capacity of the medium. Table 9 shows results of one of the better experiments. Growth was very poor at the lowest level of Na^+ (0.029 N), regardless of the K^+ level added, indicating that Na^+ was essential for growth. The results

TABLE 9

Effect of different levels of potassium and sodium on growth of Strain S85 in potassium-free basal medium with 10% CO_2—90% N_2 gaseous phase[a]

	Growth (O. D. × 100)				
Normality Na^+	0.029	0.057	0.084	0.111	0.152
Normality K^+					
0.15	0 (40)	0 (40)	0 (40)	.....	.....
0.10	4 (40)	0 (40)	0 (40)	0 (40)	.....
0.05	1 (16)	44 (31)	20 (27)	5 (27)	0 (40)
0.01	0 (40)	3 (31)	65 (27)	24 (24)	4 (16)
0.003	0 (40)	2 (31)	30 (24)	46 (27)	4 (16)
0.001	0 (40)	2 (31)	20 (27)	9 (27)	0 (40)
0.0003	0 (40)	0 (40)	0 (40)	0 (40)	0 (40)

[a] See footnotes of Table 8.

again demonstrated the essentiality of K^+. Best growth occurred with the 0.05, 0.01, and 0.003 N K^+ when 0.057, 0.084, and 0.111 N Na^+ levels, respectively, were present. Thus, at lower levels of sodium, higher levels of potassium were necessary for best growth and the reverse was also true.

The organism could be grown in a medium with sulfate salts replacing all chlorides and cysteine (free base) replacing cysteine · HCl · H_2O.

Miscellaneous experiments. To determine if *B. succinogenes* would digest cellulose in a known medium containing few ingredients, Strain S85 was inoculated into Medium C of Figure 2 modified to contain approximately 0.2% of thoroughly washed filter paper cellulose (9) in place of the glucose. The organism was carried on this medium through six daily transfers, using 0.1 ml. inoculum into 10 ml. of medium. After 24-hr. incubation of each tube a trace to about one-third of the cellulose had visibly disappeared. After 48 hr. practically all had disappeared. Thus, though considerably slower than in more complex media, growth and cellulose digestion occur in the simple, chemically defined medium with cellulose replacing glucose.

TABLE 10

Effect of various mixtures of volatile fatty acids on the growth of Strain S85[a]

Volatile fatty acid added	Casein hydrolysate	Growth (O. D. × 100)
	Experiment 1	
All[b]	Present	117 (20)
All except acetate	Present	122 (20)
Valerate + isovalerate	Present	122 (18)
Valerate	Present	0 (96)
Isovalerate	Present	1 (96)
None	Present	0 (96)
	Experiment 2	
All	Present	107 (23)
All	Deleted	117 (71)
Valerate + isovalerate	Present	105 (23)
Valerate + isovalerate	Deleted	85 (63)

[a] See Footnote [b], Table 2. The basal medium was that shown in table, except that only biotin and PABA of the B vitamins were added and the volatile fatty acids and casein hydrolysate were deleted.

[b] The fatty acids were added in the following concentrations (μM/100 ml.): acetate, 420; propionate, 150; *n*-butyrate, 100; isobutyrate, isovalerate, and DL-α-methyl-*n*-butyrate, 30 of each; and *n*-valerate, 60.

The results shown in Table 10 indicate that Strain S85 had not lost its two component volatile fatty acid requirements after being maintained in the laboratory for over 5 yr. and, contrary to studies on Strain C94 of *Ruminococcus flavefaciens* (2), acetate was not stimulatory. Also, the presence of the complete mixture of ruminal volatile fatty acids did not significantly stimulate growth above that obtained with only valerate and isovalerate, and this was true both in the presence and absence of amino acids.

DISCUSSION

In preliminary studies on vitamin requirements of Strain S85, the essentiality of PABA as well as biotin was established. None of the other B vitamins or a mixture of purines and pyrimidines (10) would replace the PABA requirement. It thus seems evident that this strain changed somewhat during cultivation on artificial medium, since PABA had no effect on growth of Strain S85, or two other strains, in the present study.

The results indicated that folic acid would not replace the PABA required for good growth of Strains S61 and CB40.

Whether biotin could be replaced by materials such as desthiobiotin, pimelic acid, or oleic acid was not determined.

The activity of biotin and PABA as growth factors for *B. succinogenes* seems to substantiate the results of Bentley *et al.* (6), who found biotin and PABA to be active in promoting cellulose digestion by washed suspensions of the mixed ruminal flora. It is of interest to speculate that the method used enriched the *B. succinogenes* of the mixture because only PABA and biotin of the B vitamins stimulated cellulose digestion. Other workers using the mixed ruminal flora have found other vitamins to be stimulatory to cellulose digestion (22, 25). Also, work on other species of ruminal cellulolytic bacteria (5, 11,

16, 17) indicate that these required some B vitamin other than, or in addition to, biotin and PABA.

It is of interest that biotin and PABA also were shown to satisfy the B vitamin requirements of some noncellulolytic ruminal anaerobes, i.e., a strain of *Lachnospira multiparus* (15) and a strain of *Bacteroides ruminicola* subsp. *brevis* (12). The latter organism is similar to *B. succinogenes* in many respects, including morphology and fermentation products, but differs in many respects, e.g., it had no volatile acid requirement but did require enzymatic hydrolysate of casein.

The very simple nitrogen requirements of *B. succinogenes,* NH_3 being the only essential nitrogenous compound, appear to be similar to those of several other ruminal bacteria. Gill and King (17) found no essential amino acids for growth of a strain of *Butyrivibrio.* Large amounts of NH_3 were assimilated, and the amount assimilated was independent of the amount of amino acid in the medium. None of the amino acids were essential for growth of *Streptococcus bovis* (28) or *L. multiparus* (15). Data of Allison (1) indicate that Strain C94 of *R. flavefaciens* will grow in a medium with NH_3 as the only source of nitrogen.

It is of considerable interest that Allison, Bryant, and Doetsch (3) found that this strain did not incorporate an appreciable amount of preformed amino acids into cellular protein during growth, but did utilize isovalerate in the synthesis of leucine. The results made it appear probable that the essentiality of isovalerate as a growth factor for *R. flavefaciens* was due to the organism's inability to assimilate preformed amino acids and its inability to synthesize the branch-chained carbon skeleton of leucine from compounds other than the branch-chained volatile acids.

It seems probable that a similar situation exists in *B. succinogenes,* which has been shown to require one of certain branch-chained, and one of certain straight-chained, volatile fatty acids for growth (10). The facts, that ammonia is essential and no amino acids are essential, tend to support this probability.

The possibility exists that the stimulatory effect of amino acids on the initiation of growth of *B. succinogenes* as shown in the present work, and on growth of a strain of *R. albus* (16) and two strains of *R. flavefaciens* (1, 5), may be due to factors not related to their incorporation into cellular protein. Among these factors might be an effect on the E_h of the culture medium or their ability to antagonize inhibitory effects of other compounds or ions in the medium.

It also is possible that ruminal organisms such as *B. ruminicola* subsp. *brevis,* which appear to require amino acids for growth (12), do not incorporate them directly into cellular protein but produce volatile fatty acids which are then used to synthesize amino acids incorporated into cellular protein. This organism is known to actively produce ammonia from protein hydrolysates. It would be of interest to determine if this organism can grow in media without amino acids if ammonia, and ruminal volatile fatty acids, were present.

Further studies on the nitrogen requirements of *B. succinogenes* and other ruminal bacteria should bring to light information regarding the ruminal metabolism of protein. It seems evident that work on nutrient requirements for growth,

as done in the present study, is not enough. Of equal importance is the determination of the actual nutrients utilized by the organism to synthesize cellular constituents, as done in studies such as those of Warner (30) and of Allison, Bryant, and Doetsch (3).

Ruminal bacteria may well be very useful tools for use in furthering basic knowledge of mechanisms involved in amino acid assimilation and synthesis of amino acids and protein.

A detailed study of the sulfur requirements of *B. succinogenes* was not possible under the conditions studied, because the only reducing agents found to be satisfactory for growth of the organism also served as sole sources of sulfur, and the level required as reducing agent was almost certainly higher than the amount needed as sulfur source.

The fact that sulfide can serve as the sole source of sulfur suggests that the organism is well adapted to life in the rumen, where sulfide appears to be the main source of sulfur that is continually present in a form available to bacteria for assimilation. It is known that appreciable amounts of sulfide usually are present in the rumen, and it is believed that the bulk of the sulfur of the rumen passes through a stage where it is present as sulfide (4, 23). Lewis (24) was unable to detect sulfur-containing amino acids in cell-free rumen fluid, though some other amino acids were detected, especially shortly after the animals were fed. It is now well established that sulfate is rapidly reduced to sulfide (23) and that cysteine and cystine is broken down with a release of sulfide (24, 29).

The fact that cysteine serves as an excellent source of sulfur for *B. succinogenes* [it does not produce sulfide from cysteine or sulfate (9)], plus the fact that sulfide is produced from cysteine or cystine by the mixed flora, seems to contradict the data of Hunt *et al.* (21), who found that cystine was a poor source of sulfur as indicated by in vitro cellulose digestion by the mixed ruminal flora. This finding might have been due to the possibility that cystine would tend to raise the E_h of the system to a point where the activity of the cellulolytic anaerobes was depressed.

Emery, Smith, and Fai To (15) studied a number of strains of ruminal bacteria for $S^{35}O_4$ incorporation during growth in complex medium. While some species showed a significant incorporation and others, none, the data on Strain S85, *B. succinogenes,* were inconclusive.

In the present work, the experiments in which attempts were made to grow the organism from large inocula in media with sulfate as the sole source of sulfur indicate that sulfate can not serve as the sole source of sulfur and/or that even large numbers of cells of the organism are unable to lower the E_h of the medium enough for growth initiation. Although neither of these points has been proved, it is almost certain that the latter assumption is true. For instance, if the organism were grown in a good medium until growth were about O.D. 0.4-0.6, and then a small amount of air admitted to oxidize the medium, growth would not resume. In work in progress on the cellulolytic cocci, similar observations have been made in strains which previous studies showed would not produce hydrogen (13). Strains which produce hydrogen appear to be much less sensitive to changes in E_h, once growth has been initiated.

The growth requirement of *B. succinogenes* for Mg^{++} and Ca^{++}, and the fact that addition of Mn^{++}, Cu^{++}, Co^{++}, and Fe^{++} was neither essential nor stimulatory, appear to be in agreement with the results of Hubbert, Cheng, and Burroughs (18), who found Ca^{++} and Mg^{++} to be slightly stimulatory to cellulose digestion by the mixed ruminal flora. The results make it seem probable that at least part of the effect of Ca^{++} noted by the above workers, and the in vivo stimulation of cellulose digestion by Ca^{++} noted by White *et al.* (31) in sheep receiving rations containing 5 % corn oil, was due to its effect on growth of the cellulolytic bacteria.

The fact that Na^+ in addition to K^+ was essential for growth of *B. succinogenes*, and the fact that higher levels of Na^+ did not increase the organisms' requirement for K^+, do not appear to be in agreement with data obtained on cellulose digestion by the mixed ruminal flora in vitro (19). However, the latter phenomenon may have been obscured by the apparently great sensitivity of the organism to variations in salt concentration.

The fact that at lower levels of Na^+ higher levels of K^+ were necessary for best growth might suggest a sparing action of one of these ions on the other. However, it is also possible that this was due to the effect of the sodium and potassium chlorides on osmotic pressure. The organism never exhibited appreciable growth when the calculated total ion concentration was outside the range of about a 0.6 to 1.2% solution of NaCl.

The essentiality of Na^+ is of considerable interest, as only certain marine bacteria and a halophile, among bacteria so far studied, have shown a need for this ion [see MacLeod and Onofrey (26)]. It is also of interest that the amounts of Na^+ and K^+ necessary for best growth of Strain S85 are similar to those of marine bacteria (26) and certain types of mammalian cells (14).

The present results indicate the relatively great ability of *B. succinogenes* to synthesize cellular constituents. The nutrition of this organism including the relatively high requirement for CO_2 (9), the requirement for volatile fatty acids (10), the essentiality of NH_3, and nonessentiality of amino acids, the ability to utilize sulfide as the sole source of sulfur, and the essentiality of Na^+, indicate how well this cellulolytic bacterium is adapted to the environment of the rumen.

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ACTIVATION AND CELL-SURFACE LOCALIZATION OF CERTAIN β-GLUCOSIDASES OF THE RUMINAL FLORA

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SUMMARY

Kinetic and inhibition studies of the cellulases in crude extracts of ruminal ingesta indicated the participation of sulfhydryl groups and polyvalent metal ions in enzymic hydrolysis of cellulose. As a result, the assay substrate has been modified by inclusion of ferric chloride. A minimum estimate was obtained of the proportion of the cellulolytic activity in ruminal ingesta which is intimately associated with bacterial cell surfaces. A series of fractionations employing washing of the bacterial cells by centrifugation, rupture in a French pressure cell, and lysis using desoxycholate yielded an estimate of 5%. Reasons for suspecting that surface-bound cellulases may occur in considerably larger proportions were discussed.

Microscopic examination of decomposing cellulosic materials freqently reveals numerous bacterial cells concentrated in the pits and crevices which appear on the surfaces of the fibers early in the course of degradation. This observation has been interpreted as presumptive evidence that intimate contact between the fiber and the cell is essential to solubilization of the substrate, perhaps as a consequence of surface-bound cellulases which can not function as freely diffusing enzymes. This interpretation, although provocative in connection with the energy economy of the cells, is highly speculative; and direct experimental evidence that certain microbial cellulases are intimately associated with the cell surface has not appeared.

The results of investigations of the localization of the β-glucosidases which initiate certain phases of the fermentation of feedstuff in ruminants have been contradictory. Kitts and Underkofler (7) initially reported that the cellulases of the rumen appeared to be localized on microbial cell surfaces. Conflicting observations have appeared from this laboratory, Gill and King (2, 3), and certain of the properties of the extracellular ruminal cellulases have been described, King (6). As a result of these latter investigations, it is evident that typical, freely diffusing, extracellular enzymes are an important component of the cellulase complex which functions in the ruminal fermentation. The substance of the present report concerns direct evidence that surface-bound β-glucosidases do function in the ruminal microflora and that sulfhydryl groups and metal ions are involved in the action of certain ruminal β-glucosidases.

EXPERIMENTAL METHODS

Assays of β-glucosidase were conducted viscosimetrically, King (6), and by measurement of reducing sugar liberation, Hash and King (5). In both assays the substrate was modified by including 10^{-3} M $FeCl_3$.

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In investigating the participation of sulfhydryl groups and metal ion activation of the ruminal β-glucosidases, Fraction 1, King (6), was used as the enzyme source. This fraction consisted of those mixed enzymes which were most rapidly eluted from whole ruminal ingesta by dilute phosphate buffer. After dialysis against running tap water, the preparation was lyophilized and stored over $CaCl_2$ at −18° C. When resuspended in dilute buffers a small amount of insoluble material remained. This residue was removed by centrifugation.

In studying localization of the enzymes on bacterial cell surfaces, the material was obtained par fistulum from steers fed a poor-quality clover–timothy hay mixture supplemented with from 2 to 4 lb. of grain concentrate per day. Samples were obtained from the lower portion of the hay mat, Smith *et al.* (11), approximately 1 hr. after the morning feeding. After squeezing the ingesta through four layers of cheese cloth, the liquid portion, hereafter referred to as rumen fluid, was taken to the laboratory in completely filled bottles.

An initial centrifugation at 1,000 × G for 10 min. at 0° C. yielded a supernatant from which most of the plant debris and protozoal cells had been removed. Further sedimentation of this supernatant at 25,000 × G for 30 min. at 0° C. packed the mixed bacterial cells tightly and resulted in a supernatant essentially free of bacteria. By careful resuspension of the upper layers of the pellet, preparations were obtained which contained neither recognizable plant particles nor protozoa in appreciable amounts. Morphologically, these suspensions were very heterogeneous, however. As a result, some question must remain concerning the absolute absence of plant residues. Gram-negative forms predominated.

Lysis of the cells was conducted in 0.05 *M* tris(hydroxymethyl)amino-methane-maleate buffer at pH 6.8 containing 50 mg. of sodium desoxycholate per milliliter, using a 15-min. treatment at room temperature (35 to 40° C.). Under these conditions, approximately 50% of the cells were lysed as estimated by the decrease in turbidity corrected for the release of quite large amounts of soluble pigments during lysis. Longer periods of treatment did not increase the amount of lysis appreciably.

Mechanical rupture of the cells was done in a French pressure cell at 12,000 p.s.i., collecting the fragmented cells in a chilled beaker. Microscopic examination of smears stained with nigrosin after a single treatment indicated fragmentation of more than 90% of the cells. Accurate estimation of the efficiency of fragmentation was not attempted because of the morphological heterogeneity of the original cell suspensions.

RESULTS AND DISCUSSION

Observation that the amount of glucose produced enzymically from carboxymethylcellulose decreased at increasing dilutions of the enzyme, when the incubation times were increased in proportion to the dilution of the enzyme, indicated a cofactor requirement. Several potential inhibitors and activators were evaluated, with the results shown in Table 1. Involvement of sulfhydryl groups is suggested by these data, but the relative stability of the enzymes to oxygen,

TABLE 1
Inhibition and stimulation of crude ruminal cellulases[a]

Test compound	Activity as per cent of unsupplemented control	Test compound	Activity as per cent of unsupplemented control
None	100	$K_2S_2O_3$	74
Metallo-enzyme inhibitors		$MnCl_2$	85
NaCN	36	$NH_2OH \cdot HCl$	81
NaN_3	99	$NaHAsO_3$	82
α,α-Bipyridyl	66	$FeSO_4$	100
o-Phenanthroline	100	Bovine serum albumin	100
Sodium EDTA	94	$MgCl_2$	106
Sulfhydryl inhibitors		$CaCl_2$	111
p-CMB	88	Li_2SO_4	116
Iodoacetamide	57	KCl	117
$HgCl_2$	21	$BaCl_2$	122
$AgNO_3$	86	$SnCl_2$	124
$Pb_2(C_2H_3O_4)_2$	95	Cysteine	132
Other compounds		KI	132
$UO_2(NO_3)_2$	75	Na_2HAsO_4	132
Na_2S	35	$FeCl_3$	138
$ZnCl_2$	96	$COCl_2$	141
$CuCl_2$	70		

[a] Reaction mixtures consisted of 0.5 ml. of enzyme (0.5 mg. of crude powder) in water, 0.5 ml. of water or test compound (final concentration $= 10^{-3}$ *M*), plus 1.0 ml. of assay substrate. Incubation was at 37° C. for 2 hr. Activity was measured in terms of glucose equivalents produced per tube, corrected for both the heat-inactivated enzyme control and for the color produced by each test material in the absence of enzyme.

Gill and King (2), implies that the sulfhydryl groups are somewhat protected. Basu and Whitaker (1) reported a similar sensitivity of purified cellulase from *Myrothecium verrucaria* to inhibitors of sulfhydryl-dependent enzymes. The stimulatory effect of serum albumin which Whitaker (14) reported was not evident in the present system, perhaps because only soluble substrates have been used in the present study.

The inhibitory action of cyanide, ethylenediaminetetraacetate, and α,α-bipyridyl, and the apparent activation by ferric and cobaltous ions seen in Table 1 were examined further. Both $CoCl_2$ and $FeCl_3$ reversed the inhibitions by ethylenediaminetetraacetate and α,α-bipyridyl at 10^{-3} *M*. In addition, the data in Figure 1 demonstrate that $FeCl_3$ alone prevented the decrease in product formation as decreasing concentrations of enzyme were incubated for inversely proportional times. The specificity of the metal ion requirement has not been pursued further, since methods for separating the several enzymic components known to be present in the crude preparation have not been developed. All subsequent assays, however, were carried out in reaction mixtures containing 10^{-3} *M* $FeCl_3$, there being no information bearing on what proportion of the ruminal cellulases is activated by trivalent iron and no indication of inhibition by iron.

Using the viscosimetric assay, readily detectable amounts of enzyme were observed in whole cell suspensions washed as many as eight times in large volumes of either 0.05 *M* phosphate or tris(hydroxymethyl)aminomethane-maleate buffer at pH 6.8. In a typical experiment, 8.0 ml. of rumen fluid was subjected to the low- and high-speed centrifugations described earlier. After discarding the supernatant from the high-speed centrifugation, the pellet was resuspended in 8.0 ml.

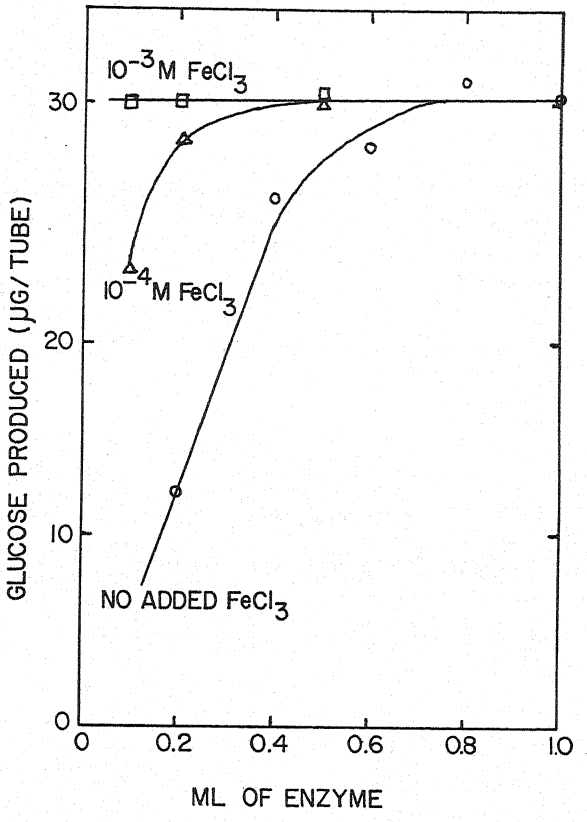


FIG. 1. Reversal by iron of the reduction in enzyme activity as a result of dilution. The concentration of enzyme (milligram/milliliter) times the time of incubation (minutes) in all instances = 30 mg.-min. per milliliter.

of buffer and centrifuged at 25,000 × G for 30 min. at 0° C. The resulting supernatant was then assayed. This washing procedure was repeated three more times, assaying each supernatant and the final pellet. The total recovery of enzyme in the successive supernatants was 175, 145, 44, and 30 units, and in the final resuspended whole cells, 122 units. With a second, similar sample of rumen fluid the successive washes contained 88, 26, 30, and 17 units, and the cells contained 131 units.

Microscopic examination of the washed cell suspensions indicated the presence of some plant residues having centrifugal properties similar to those of the bacteria. The possibility existed that the activity of the washed cells represented enzymes adsorbed on the cellulosic residues, the enzymes being desorbed when added to the soluble assay substrate. That this was not so was indicated by control experiments, in which parallel cell preparations were made using assay substrate as the washing medium in one preparation and the assay buffer components as the washing medium in the other. In a typical experiment involving four washing steps, the buffer-washed cells retained 138 units of enzyme com-

pared to 145 units in the cells washed with carboxymethylcellulose, the difference being insignificant. These results indicate that the activity is associated with the bacterial cells themselves.

If the activity of washed cell suspensions were attributable to localization of the enzymes on the surface of the cells, two observations should be possible. First, lysis of the cells should be associated with release of the enzymes into solution, although either incompleteness in solubilization of the cell wall or denaturation of the enzyme might cause the quantitative correlation between lysis of cells and solubilization of enzyme to be approximate. Second, nonlytic fragmentation of the cells using the French pressure cell should yield enzymically active particles (cell wall fragments) from which the enzyme could be released later by lysis. As a corollary, the amount of enzyme retained by the cell wall fragments after treatment in the pressure cell but solubilized by subsequent lysis should serve as a minimum estimate of the amount of surface-localized cellulase in the ruminal flora.

Estimating lysis turbidimetrically after 1-hr. incubation periods, less than 5% lysis was obtained using lysozyme (2 to 200 γ/ml) alone, lysozyme followed by treatment with NH_4OH, lysozyme following pretreatment of the cells with acetone, Warren *et al.* (12), or lysozyme in conjunction with ethylenediaminetetraacetate, Repaske (10), 0.25 *M* glycine, 0.25 *M* NaCl, or 0.5 mg/ml of trypsin. Cells suspended at 0° C. for 15 min. in saturated NaCl, harvested by centrifugation, and then resuspended in distilled water likewise failed to lyse significantly. Polymyxin-B sulfate at concentrations between 8.25 and 125 γ/ml, followed by treatment with lysozyme at concentrations of 3 to 30 γ/ml, as described by Warren *et al.* (13), failed to produce appreciable lysis. Saponin, bile salts, and sodium-desoxycholate lysed the preparations rapidly. It was also found that under the conditions used here desoxycholate did not influence the amount of soluble ruminal cellulase adsorbed onto powdered cellulose.

A whole cell suspension was prepared from 64 ml. of rumen fluid as described previously and washed three times in 8 ml. of 0.05 *M* tris(hydroxymethyl)aminomethane-maleate at pH 6.75. Use of this buffer was dictated by the fact that on mixing phosphate and sodium desoxycholate, a thixotropic gel which invalidated viscosimetric assays was formed. The cells were resuspended in 20 ml. of the same buffer and filtered through glass wool to remove poorly dispersed clumps. One-milliliter portions of the cell suspension were then incubated for 15 min. at room temperature with 1.0 ml. of sodium desoxycholate at several concentrations between 0 and 75 mg/ml. Initial and final turbidity measurements were recorded, using the Klett-Summerson colorimeter with the blue filter. After centrifugation at 29,000 × G for 20 min. in the cold, the supernatants were assayed viscosimetrically for cellulase and the optical density at 380 mμ was measured in a Spectronic-20 colorimeter and in the Klett-Summerson colorimeter using the blue filter. At this wave length absorption by the post-lysis, supernatant was maximum. The optical density data using the two instruments were proportional. To correct for the release of soluble pigments

during lysis, the per cent lysis was calculated as follows: per cent lysis = 100

$$\left[\frac{\text{O.D. before lysis} - (\text{O.D. after lysis} + \text{O.D. of the supernatant})}{\text{O.D. before lysis}}\right]$$

The results are summarized in Figure 2. It is evident that the amount of enzyme solubilized during lysis of the cells was closely related to the amount of lysis. Assay of the original cell suspension indicated 10.8 units of enzyme per milliliter. Those preparations lysed with 50–75 mg. of desoxycholate per milliliter yielded supernatants containing 42.8 to 46.4 units of enzyme per milliliter. The increase in activity as a result of lysis does not appear to be an artifact and will be referred to later.

Assessment of the quantitative significance of the cell-bound cellulases was attempted. In this experiment, the amount of enzyme in each fraction at each step was determined by assay. A 400-ml. portion of rumen fluid was centrifuged at 1,000 × G for 10 min. at 0° C. The resulting supernatant was then centrifuged at 25,000 × G for 30 min. The resulting pellet was resuspended in 50 ml. of 0.05 *M* tris(hydroxymethyl)aminomethane-maleate at pH 6.75 and washed by a second high-speed centrifugation. Washing of the pellet was repeated four more times. Half of the final cell suspension was then lysed under the usual conditions in 50 mg. of sodium desoxycholate per milliliter. The lysed suspension was then centrifuged at high speed, yielding a cell-free supernatant and a particulate residue. The other half of the final cell suspension was treated in a French pressure cell and separated into soluble and particulate components

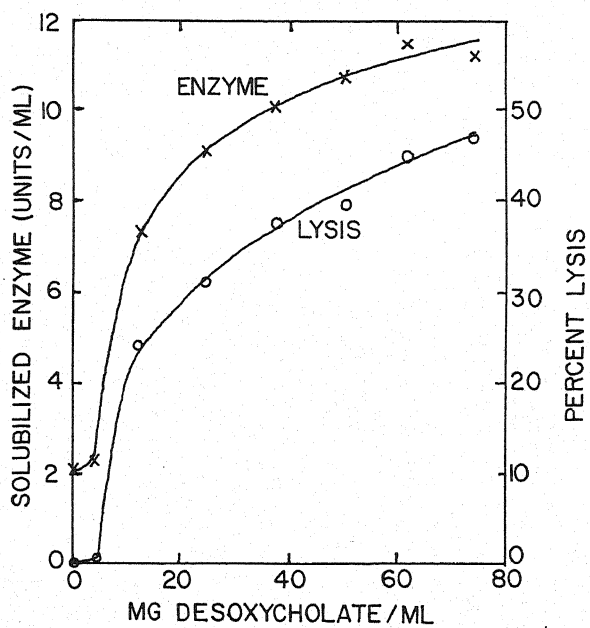


FIG. 2. Lysis and solubilization of particulate enzymes as a result of treatment with desoxycholate.

by high-speed centrifugation. The particulate fraction was resuspended in buffer, lysed using 50 mg. of sodium desoxycholate per milliliter, and centrifuged again at high speed. The results are summarized in Table 2.

The assumption was made that the activity retained in the particulate fraction after treatment in the pressure cell, and later released in soluble form as a result of lysis, gives a minimum estimate of the surface-bound bacterial cellulase. This fraction, the supernatant of Step 10, accounts for approximately 5% of the total activity in the original sample. Several factors indicate that this value is a minimum estimate. In preparing the cell suspensions, emphasis was placed on elimination of plant residues and protozoa rather than on quantitative recovery of bacteria. Some degree of lysis is likely to have occurred during preparation of the cell suspensions. Lysis of the cell suspensions, using desoxycholate at a concentration of 50 mg/ml, was only approximately 50% complete.

The quantitative discrepancies which appear in this experiment can be explained in certain instances. Reliable corrections, however, can not be made because of existing gaps in specific information concerning the enzyme system under study.

The occurrence in ruminal ingesta of substances inhibitory to cellulases has been encountered repeatedly in this laboratory, King (6). Very little is known of either the chemical or the enzymological properties of these inhibitors. The high recoveries seen in the early centrifugal steps (2 through 6) of the experiment summarized in Table 2 probably resulted in part from progressive removal of inhibitors and in part from partial lysis with release of soluble intracellular enzymes.

The increase in the amount of assayable cellulase (Figure 2) which accompanied lysis with desoxycholate may also bear on the excessive recoveries in Steps 2 through 6. A truly intracellular β-glucosidase which is capable of degrading cellulosic substrates of relatively high degree of polymerization has been described by Youatt (15). Considering the extreme heterogeneity of the cellulolytic ruminal flora, participation of similar intracellular enzymes in the utilization of cellulose as a source of energy must be entertained as a possibility. No measure of the amount of lysis which occurred in the early stages of the fractionation was made. The unusual resistance of the final cell suspensions to lytic processes, however, suggests that many fragile cells may have lysed during washing of the cells, thus releasing normally intracellular β-glucosidases into the suspending medium.

It is probable that the assay values in Steps 9 and 11, and of the supernatants in Steps 10 and 12, were approximately 10% low, because control experiments showed that desoxycholate was inhibitory to that extent under the conditions used.

Caution in rigid interpretation of the quantitative aspects of the data in Table 2 is also dictated by the fact that the assay measures the activity of a group of enzymes differing in physical properties, specificity, and mode of action, Reese (9), King (6), Grimes *et al.* (4), Hash and King (5), and Miller and Blum (8). The validity of the assay was established for the extracellular

TABLE 2

Fractionation of ruminal cellulases by centrifugation, lysis, and rupture in a pressure cell to access the proportion which is surface-bound

Step No.	Treatment[a]	Fraction	Use (refer to Column 1)	Total activity[b] (units of cellulase)	Per cent recovery for each step[c]
0	None	Original sample	Step 1	16,000	.....
1	Low-speed centrifugation	Sediment	Discarded	2,930	
		Supernatant	Step 2	12,400	96
2	High-speed centrifugation	Sediment	Step 3	2,700	
		Supernatant	Discarded	16,200	152
3	High-speed centrifugation	Sediment	Step 4	2,250	
		Supernatant	Discarded	395	98
4	High-speed centrifugation	Sediment	Step 5	2,770	
		Supernatant	Discarded	472	144
5	High-speed centrifugation	Sediment	Step 6	2,460	
		Supernatant	Discarded	635	112
6	High-speed centrifugation	Sediment	Steps 7 and 11	2,340	
		Supernatant	Discarded	222	104
7	Rupture in pressure cell	.....	Step 8	4,080	174
8	High-speed centrifugation	Sediment	Step 9	1,350	
		Supernatant	Discarded	3,470	118
9	Lysis with desoxycholate	.....	Step 10	1,450	107
10	High-speed centrifugation	Sediment	Discarded	618	
		Supernatant	Discarded	842	101
11	Lysis with desoxycholate	.....	Step 12	5,470	234
12	High-speed centrifugation	Sediment	Discarded	1,010	
		Supernatant	Discarded	3,990	92

[a] Low-speed centrifugation was carried out at 1,000 × G for 10 min. at 0° C.; high-speed centrifugations were carried out at 25,000 × G for 30 min. at 0° C.

[b] All values have been corrected to indicate the amount of enzyme on the basis of the original sample of ruminal ingesta.

[c] These values represent the sum of the activities in each pair of fractions (i.e., supernatant plus sediment) expressed as per cent of the activity in the starting material for that step.

cellulases of the ruminal flora, and was assumed in the present studies, in so far as particulate cellulases were concerned.

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EFFECTS OF DIETARY DIETHYLSTILBESTROL ON SEVERAL BIOCHEMICAL FACTORS OF RUMEN INGESTA[1]

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SUMMARY

Two pairs of rumen-fistulated identical twin cows were used to determine some of the effects of low-level feeding of diethylstilbestrol (DES) on the rumen microflora. One twin of each pair was fed 10 mg. of DES per 1,000 lb. of body weight daily. Rumen ingesta samples were collected and determinations were made on pH, surface tension, total free volatile fatty acid concentration, percentage acetic, propionic, and butyric acids, in vitro gas production, and in vitro cellulose digestion as indirect indications of rumen microorganism activity.

DES did not cause a marked change in any of these measurements. However, there was a trend toward decreased in vitro cellulose digestion in the miniature artificial rumina inoculated with rumen liquor from cows receiving DES in their ration. The addition of 20 μg. of pure DES in vitro had no effect on cellulose digestion in the artificial rumina that had been inoculated with rumen liquor from the control cows, but was slightly stimulatory to cellulose digestion when inoculated with liquor from the treated cows.

It is concluded that the oral administration of DES by the addition of "Stilbosol" to the feed in amounts to furnish 10 mg. per 1,000 lb. of body weight daily did not have a marked effect on the microbial activity in the rumen of the cows studied.

The effects of diethylstilbestrol (DES) on the digestibility of nutrients in the rations of ruminants have been reported by many workers, but the effects on rumen contents have been less thoroughly investigated. Several groups of workers (1, 9, 14, 15) reported that the inclusion of DES in the ration of ruminants did not significantly affect digestibility. Story *et al.* (18) reported that the digestibility of dry matter of a ration fed to lambs was increased by feeding 2 to 4 mg. of DES daily. Increases in the coefficients of digestion of cellulose and protein in the rations of wether lambs fed 10 to 20 mg. of DES daily also have been reported (3). The object of this study was to determine whether low levels of DES fed daily to cattle would affect the rumen microflora as measured by biochemical factors.

EXPERIMENTAL PROCEDURE

Animals and rations. Two pairs of identical twin cows were used in this study. Each animal had a permanent rumen fistula fitted with a plastic cannula and cap. The cows were fistulated during the latter half of pregnancy and calved without difficulty. Both pairs of twins (81-82 and 31-32) were in their third lactations and were not bred during the experiment. One animal of each pair

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was fed the experimental ration containing DES and its twin was fed the control ration. In Twin Pair 81-82, Cow No. 82 was fed the experimental ration, since this animal had received it in the two preceding lactations. In the second pair (31-32) Cow No. 31 was chosen at random to receive the experimental ration. The cows were milked twice a day, at 6 A.M. and 6 P.M.

Feeding methods. A modified paired-feeding technique was used throughout the experiment. The cows were fed chopped alfalfa hay at a rate so that there was a small daily weigh-back. Concentrates were fed at a rate of approximately 1 lb. for each 4.0 lb. of 4% fat-corrected milk produced daily during the first 2 wk. of lactation. The amount fed was the same for each twin within a pair. The rations were fed at milking time.

DES was furnished in Stilbosol,[3] a concentrate containing 1 g. of DES per per pound. Sufficient Stilbosol to furnish 5 mg. of DES per pound of concentrate mixture was used as a replacement for part of the concentrate mixture of the treated animal of each pair. The formulas for the concentrate mixtures (14% total protein) were as follows:

Control concentrate mixture		DES-containing mixture
	(lb.)	*(lb.)*
Ground corn	400	400
Ground oats	200	200
Soybean oil meal	100	96.5
Salt	7	7
Steamed bone meal	7	7
Stilbosol	.....	3.5

The quantity of feed offered and the quantity refused were carefully weighed and recorded. In general, the ration was consumed in 2 hr. Water was available at all times in automatic watering cups.

Biochemical methods. Five 12-hr. trials were used for each pair of twins. Two of these trials served as a preliminary period for each pair before the start of DES feeding. Rumen samples were taken four times during each trial, at 5 and 10 A.M., and 2 and 4 P.M.

The water in the automatic drinking cups was turned off 1 hr. before each sampling. The 5 A.M. and 2 P.M. samples were taken from the rumen via the fistula, after the rumen ingesta had been mixed by hand for approximately 3–4 min. The 10 A.M. and 4 P.M. samples were taken after the entire rumen contents had been removed, weighed, and thoroughly mixed in a large tub. The emptying, weighing, sampling, and refilling of each rumen was done as rapidly as possible; usuallly, less than 30 min. was required. During the first four trials for each pair, pH and surface tension were determined on the 5 A.M. and 2 P.M. samples; whereas, during the last trial, these determinations plus those for volatile fatty acids (VFA) were made. The 10 A.M. samples were checked for pH, surface tension, VFA content, and total residual gas production from a 200-g.

[3] Supplied by Eli Lilly and Company, Indianapolis, Indiana.

sample. The 4 P.M. samples were checked for pH, surface tension, and VFA content. All samples were transported from the barn to the laboratory in glass screw-cap bottles. The samples were protected to prevent temperature change.

pH. A Beckman potentiometer was used for the determination of pH of the rumen samples. The pH readings were made on the rumen samples as soon as they were brought to the laboratory.

Surface tension. Rumen liquor, resulting from the squeezing, by hand, of rumen ingesta, was brought to the laboratory and allowed to stratify while being brought to 27° C. in a water bath. After a period of 30 to 45 min., the sample had stratified to such an extent that the straw-colored, clear layer could be strained through cheese cloth. The resulting liquor was used for surface tension determinations, using a du Nouy tensiometer. The average of three readings was recorded.

Free volatile fatty acids (VFA). Samples of liquor squeezed from the ingesta by hand were rapidly cooled, frozen, and held at approximately −23° C. until thawed for VFA determinations. The samples were thawed rapidly, but in a manner permitting the sample temperature to remain fairly cool.

Acetic, propionic, and butyric acids were separated by direct chromatography of rumen liquor on silica gel in a manner similar to that used by Mueller *et al.* (16).

The thawed rumen sample was mixed thoroughly and approximately 2 ml. clarified by centrifuging (2,000 G). One milliliter of the clarified sample was acidified with 1 ml. of 0.5 *N* sulfuric acid. One milliliter of the resulting acidified sample was added to 2 g. of washed and dried silicic acid, thoroughly mixed, and added to the top of the column after chloroform had been added to form a slurry.

Results of preliminary work indicated that the acid fraction reported as butyric contained traces of valeric acid. Other workers (8), using a similar technique, reported that the butyric acid fraction contained traces of higher acids; therefore, it was assumed that the fraction reported as butyric in the present study contained traces of higher acids present in the rumen liquor.

To determine the total quantity of VFA in the rumen, composite samples of 400 to 600 g. of rumen ingesta were collected at the 10 A.M. and 4 P.M. samplings, accurately weighed to the nearest gram, and dried in a forced air oven at 79° C. for 24 hr. The percentage of liquid present in the sample was calculated and applied to the total weight of rumen ingesta, to arrive at the quantity of liquid in the rumen. The total quantity of VFA present in the rumen was then calculated.

Gas production. Two-hundred-gram samples of rumen ingesta were collected at the 10 A.M. sampling for determining the total amount of gas produced. Samples were weighed into quart jars, placed in a constant temperature (39 ± 1° C.) water bath, and connected by a rubber tube to a gas-collecting cylinder. The gas-collecting vessel consisted of a 450-ml. calibrated glass cylinder containing water.

During a preliminary trial, a saturated salt solution (13) was used in the cylinders instead of water, and CO_2 was used to sweep the air out of the fermentation flasks before they were stoppered and transported to the laboratory. Hungate *et al.* (12) reported that there was no apparent difference in the mi-

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crobial activity between rumen ingesta that were gassed with CO_2 before transporting to the laboratory and ingesta that were not gassed.

Neither using the saturated solution nor gassing the sample with CO_2 appeared to change the amount of gas produced and, consequently, neither procedure was used after the preliminary trials.

During the last two trials (IV and V), a solution of DES was added to two of the four fermentation flasks containing ingesta from each animal to determine the effect of DES on gas production in vitro. Forty μg. of pure DES was added to the 200-g. sample of rumen ingesta in Trial IV, and 80 μg. was added in Trial V.

Cellulose digestion (in vitro). The miniature artificial rumen described by Hanold *et al.* (11) was used to determine the effect of DES on cellulose digestion in vitro.

Samples of rumen liquor were collected at 3 P.M. The rumen contents were stirred, as previously mentioned, and the rumen liquor was squeezed through four thicknesses of cheese cloth and transported to the laboratory with a minimum of heat loss. In each artificial rumen was placed 500 mg. of finely ground alfalfa as substrate and 10 ml. of strained rumen liquor as inoculum. The ground alfalfa hay contained 29% cellulose. One trial, using 24 miniature artificial rumina, was conducted. Six miniature rumina were inoculated with the liquor from each cow. Two of the six for each cow contained 10 μg. of DES, two contained 20 μg. of DES, and the remaining two contained only the substrate and inoculum. Pure DES was used for all in vitro studies.

The contents of the miniature rumina were incubated for 24 hr. in a constant temperature water bath at 39 $\pm$ 1° C. At the end of the fermentation period, microbial activity was stopped by the addition of ethanol. Cellulose determinations of the contents of each miniature rumen were made, employing the method of Crampton and Maynard (7). The difference in the amount of cellulose at the start and that remaining after fermentation was considered to be the amount digested. To determine the amount of cellulose at the start, 10 ml. of rumen liquor from each animal was added to a beaker containing 500 mg. of ground alfalfa hay. Fermentation was prevented by the addition of ethanol. Cellulose determinations were then made on these samples at the same time as those that had been digested.

RESULTS

pH. The mean pH values of rumen ingesta measured in vitro of each of the four daily samplings during the preliminary (Trials I and II) and test periods (Trials III, IV, and V) are shown in Table 1. It is evident from the uniformity of the pH values between twins within a pair that the feeding of DES at low levels did not measurably affect the pH of the rumen ingesta. There was a change in the average pH between the preliminary and test periods; however, this difference was evident in both treated and control animals of both twin pairs.

In general, there was greater uniformity of pH readings between twins within any one trial than there was between the readings for the same cow among the various trials. Since the animals were fed and managed as nearly as possible

TABLE 1
Effect of DES on pH of rumen contents collected at four periods during the day from identical twin cows

Trial No.	Cow No.	DES fed daily/ 1,000 lb. body wt.	Mean pH value of rumen ingesta				Mean for Trials (1 and II) and (III, IV, and V)
			5 A.M.	10 A.M.	2 P.M.	4 P.M.	
		(mg.)					
I and	81	0	6.42±.10[a]	6.08±.32	6.30±.07	6.52±.19	6.33
II	82	0	6.44±.10	6.11±.37	6.40±.20	6.60±.14	6.38
III, IV,	81	0	6.31±.33	5.92±.12	6.20±.10	6.47±.06	6.23
and V	82	10	6.37±.17	6.03±.07	6.33±.06	6.52±.06	6.31
I and	32	0	6.37±.00	6.07±.00	6.36±.07	6.50±.10	6.41
II	31	0	6.78±.00	6.03±.12	6.43±.00	6.56±.00	6.45
III, IV,	32	0	6.72±.00	6.17±.07	6.52±.04	6.62±.00	6.51
and V	31	10	6.77±.04	6.28±.04	6.58±.06	6.72±.04	6.59

[a] Standard error of the mean.

in the same manner throughout the experiment, the lack of uniformity between trials is evidence of the marked influence of environment.

Surface tension. In Table 2, a summary of the data collected on the surface tension of strained rumen liquor is presented.

There is not a uniform trend in the changes of the surface tension readings between the preliminary period and the treatment period for either pair of twins. The average of samples during the preliminary period and test period for Cow No. 81 of 53.7 and 54.7 dynes/cm, respectively, showed a trend toward slightly higher surface tension during the test periods. Rumen samples from Cow No. 82 showed the opposite trend in surface tension, decreasing from 56.1 dynes/cm in the preliminary period to 54.8 dynes/cm during the period of DES feeding. In the other pair of identical twins (31-32), the changes were: 54.9 to

TABLE 2
Effect of DES on average surface tension values for rumen liquor collected at four periods during the day from identical twins

Time of sampling	Mean surface tension of strained rumen liquor			
	Preliminary period	Test period	Preliminary period	Test period
	(dynes/cm)			
	No DES		DES[a]	
	81		82	
5 A.M.	52.5	53.8	52.4	54.3
10 A.M.	52.4	54.1	56.2	54.8
2 P.M.	54.7	55.3	58.5	55.4
4 P.M.	55.3	55.4	57.3	54.8
Average	53.7	54.7	56.1	54.8
	32		31	
5 A.M.	51.3	53.8	53.7	53.7
10 A.M.	55.9	51.8	51.7	52.6
2 P.M.	55.5	51.9	56.4	54.6
4 P.M.	56.7	52.8	56.7	53.8
Average	54.9	52.6	54.6	53.7

[a] 10 mg. stilbestrol/1,000 lb. of body weight daily.

52.6 dynes/cm for treated Cow No. 31 and 54.6 to 53.7 dynes/cm for the control, Cow No. 32.

The surface tension of the rumen liquor was fairly constant for all of the animals studied. The mean and standard deviations in dynes/cm, of 20 determinations on liquor from each cow during the preliminary and test periods, were as follows: Cow No. 81—54.2 ± 2.6; No. 82—55.3 ± 2.3; No. 31—54.0 ± 2.1; and No. 32—53.5 ± 3.1. These values were in general agreement with those reported by others workers (2, 19), and the differences were not significant.

Free volatile fatty acids (VFA). The mean quantities of VFA found in the rumen ingesta during the five trials of this study are shown in Table 3.

TABLE 3

Effect of DES on average values for pH, total free volatile fatty acids (VFA), and the percentage of acetic, propionic, and butyric acids present in the rumen of identical twin cows

Trial No.	Cow No.	Time	pH	VFA present: Total in rumen content	VFA present: mM/100 ml. rumen liquor	VFA present: Acetic	VFA present: Propionic	VFA present: Butyric
				(M)		(%)		
	81	10 A.M.	6.08	3.67	14.25	61.2	18.7	20.6
I		4 P.M.	6.52	1.63	9.05	60.3	17.8	21.9
and								
II	82	10 A.M.	6.11	2.66	15.41	61.6	18.3	19.7
		4 P.M.	6.60	1.44	8.58	64.2	15.3	20.6
III,[a]	81	10 A.M.	5.92	6.89	16.08	68.8	15.3	15.9
IV,		4 P.M.	6.47	4.12	10.84	68.7	15.8	15.5
and								
V	82	10 A.M.	6.03	5.83	14.79	68.5	16.2	15.4
		4 P.M.	6.52	3.52	9.40	64.6	16.9	18.9
	32	10 A.M.	6.07	4.53	12.98	68.5	16.4	15.2
I		4 P.M.	6.50	2.23	7.70	69.4	14.5	15.9
and								
II	31	10 A.M.	6.03	5.09	13.91	68.8	16.4	14.8
		4 P.M.	6.56	1.73	6.82	74.4	13.4	12.2
III,[a]	32	10 A.M.	6.17	4.85	10.14	67.8	16.5	15.8
IV,		4 P.M.	6.62	3.33	9.00	67.1	15.6	17.4
and								
V	31	10 A.M.	6.28	4.80	10.40	68.8	15.9	15.3
		4 P.M.	6.72	3.05	8.26	62.2	16.8	21.0

[a] Cows No. 82 and No. 31 received DES during Trials III, IV, and V (10 mg/1,000 lb. body wt/day).

During the preliminary period, the rumen ingesta from Cow No. 81 at the 10 A.M. samplings contained an average of 3.67 *M* of VFA, whereas the ingesta from Cow No. 82 contained only 2.66 *M;* however, this difference is not found in a comparison of the VFA per 100 ml. of rumen liquor. As shown in Table 3, these values were 14.25 and 15.41 m*M*/100 ml. of rumen liquor for Cows No. 81 and 82, respectively. The reason for this comparatively large difference in total moles of VFA was the difference in total weight of rumen contents of the twins during the first two trials. These observations point out the advantage

of knowing both the relative amounts of VFA in the rumen ingesta and the total weight of the ingesta. The weight of the rumen contents of these two cows was fairly close during Trials III, IV, and V, so valid comparisons can be made between Cows No. 81 and 82, using total moles of VFA and m*M*/100 ml. of rumen liquor. This comparison revealed that during the period of DES feeding, the control cow (81) had an average of 1.06 *M* (1.29 m*M*/100 ml.) more of VFA in the samples of ingesta studied at the 10 A.M. sampling than did the treated cow, No. 82.

The concentrations of VFA of Twin Pair 31-32 were similar throughout the experiment. The rumen content weights also were similar for these two cows, so that an accurate comparison between m mol of VFA/100 ml. of rumen liquor could be made. At the 10 A.M. sampling, Cow No. 31 had an average of 0.93 m*M*/100 ml. more of rumen liquor than did Cow No. 32. The difference in m mol of VFA of these two cows during the period of DES feeding was only 0.26. The average total VFA concentration in moles of acid during the period of DES feeding was 4.85 and 4.80 for 32 and 31, respectively.

The average percentages of each of the three acids separated (acetic, propionic, and butyric) were similar when compared for twins of a pair for the same period of time (Table 3).

The quantity of concentrate fed to Cows 81 and 82 was increased approximately 50% after Trial II. This increased concentrate feeding, plus an increased hay consumption, was apparently the reason for the higher total VFA concentration in the rumen contents of both cows, No. 81 and 82, during the last three trials.

Gas production. The volume of gases evolved from 200-g. samples of rumen ingesta fermented in vitro was recorded at the end of 1, 2, 3, 5, and 7 hr. The collected gases were released after each reading, and the average of the readings made on four separate gas-collection units for each animal were compared. There was good agreement among these readings of the four replicas.

The total gas evolved during the fermentation process of Trials I and II for Twin Pair No. 81-82 was greater than that from either animal during Trials III, IV, or V. The reason for this is unknown. However, as shown by the data of these studies, the fermentation processes can not be expected to remain constant from one period of time to another, even though the feeding regime remains unchanged.

Although the gas production is not the same for each of the treated animals as for its pair-mate, there is no uniform trend in the differences noted. The average total gas evolved during the 7-hr. fermentation period from the ingesta of Twin Pair 81-82, respectively, was 541 and 557 cc. during the preliminary period, and 384 and 373 cc. for the test period. The gas production results for Twin Pair No. 31 and 32 were more uniform throughout all of the trials. During the preliminary period, the rumen ingesta from Cow No. 32 produced more gas in both trials. The average total gas for Cows No. 31 and 32 during the preliminary period was 420 and 447 cc., respectively. During the period of treatment the ingesta from No. 31 gave more total gas during fermentation in all three

trials, with an average of 421 cc. as compared to 413 cc. for the control cow, No. 32.

The addition of DES to the fermenting ingesta from all four animals did not caues any marked change in the amount of gas evolved.

Cellulose digestion (in vitro). The results of the effect DES had on cellulose digestion are shown in Table 4.

TABLE 4
Effect of DES on in vitro cellulose digestion

Contents of artificial rumina		Cow providing inoculum			
		81	82[a]	32	31[a]
		Cellulose digested			
		(%)			
Basal[b] (Control)	Av.[d]	55.4±1.5[e]	51.6±0.5	54.3±0.4	53.1±1.5
Basal + 10 μg. DES[c]	Av.	55.2±0.6	53.2±1.0	54.6±0.4	53.2±1.4
Basal + 20 μg. DES	Av.	55.4±0.2	56.2±1.1	54.7±0.3	54.6±0.9

[a] Cows No. 82 and 31 were receiving DES (10 mg/1,000 lb. body wt/day) in their ration at the time the rumen liquor was collected for this experiment.
[b] Basal indicates ground alfalfa hay.
[c] Pure DES added in vitro.
[d] Average of digestion in two artificial rumina.
[e] Standard error of mean.

Since Cows No. 82 and 31 were receiving DES in their daily ration, their rumen contents probably contained it; consequently, the control part of the experiment was also a test of the effect of feeding DES on in vitro cellulose digestion. When miniature artificial rumina were inoculated with rumen liquor from cows receiving DES in their ration, there was a slight decrease in the per cent of cellulose digested, as compared to the amount digested by inoculum from the two cows receiving no DES in their ration. The addition of 10 μg. of DES to the artificial rumina did not cause a marked change in the amount of cellulose digested. However, when 20 μg. of DES was added to the artificial rumina inoculated with rumen liquor from each cow, there was a small increase in the percentage of cellulose digested in vitro when the inoculum was obtained from treated cows. However, there was little or no change in the percentage of cellulose digested upon the addition of DES in vitro when the inoculum was obtained from untreated cows. The increase in cellulose digestion in the case of the treated cows was not of sufficient magnitude to result in an increase over the controls, but merely raised the quantity digested into the general range noted for the control cows.

Although the differences in the percentage of cellulose digested were small, the data indicate that in vitro cellulose digestion is affected by DES.

DISCUSSION

That orally administered DES stimulates growth in beef cattle and sheep is a well established fact, and although there is disagreement among the workers in the field, it has been reported by several authors (5, 10) to stimulate milk production. The method by which these beneficial effects come about is not

established. It is generally thought that the nutritional benefit the bovine receives from the feeding of DES is the result of (1) the effect it has in the rumen or (2) its action upon the endocrine system. The latter of these two possibilities seems to be the more widely accepted. However, the reports of several authors (3, 18) indicate the possibility of a local ruminal effect of DES. Furthermore, Pfander *et al.* (17) concluded that orally administered DES had a beneficial effect on the utilization of nutrients through its action on the rumen microflora.

The results of the present study do not support the suggestion that the beneficial action of DES is due to its effect on rumen microorganisms.

As far as can be determined from the data collected, the feeding of low daily levels of DES to lactating dairy cows did not measurably influence (1) the pH of rumen ingesta, (2) the surface tension of strained rumen liquor, (3) the concentration of VFA in rumen ingesta, (4) the average percentage of each of the three major VFA, or (5) the total quantity of gas evolved during a 7-hr. in vitro fermentation of rumen ingesta. It seems likely that if DES had a marked effect (i.e., enough to cause a stimulation to growth or milk production by increased utilization of nutrients) upon the microflora of the rumen, some indication of it would have been observed during the course of the reported experiments. The only indication of influence exerted by DES on the microflora at the level fed was noted in the study of in vitro cellulose digestion.

The results indicate a trend toward lowered in vitro cellulose digestion by the rumen microorganisms from the two identical twin cows receiving DES in their ration, when compared to their respective pair-mates. This indication of decreased microbial activity was not noted in any of the other tests designed to establish relative microbial activity.

The report of Brooks *et al.* (3), which indicated that the addition of DES to the artificial rumen increases cellulose digestion, was not substantiated by the results of this study.

It seems from the results of these in vitro cellulose digestion studies that the decrease in cellulose digestion noted in the artificial rumina inoculated with rumen liquor from the treated cows is offset by the addition of 20 μg. of DES to the substrate. The implication of these observations is not fully understood. However, it might indicate that levels of DES higher than those used in this study are stimulatory to in vitro cellulose digestion.

From the data presented, it appeared that low level feeding of DES did not have a marked effect on rumen microbial activity. This would suggest that the beneficial response obtained in many studies in which low levels of DES were fed is a result of the action DES exerts after reaching the systemic circulation via the portal blood stream. However, since a lack of marked effect on some of the endocrine glands from animals fed low daily levels of DES has been reported (4) and increased nitrogen retention in DES-treated animals has been widely reported (16, 18, 20), it is possible that the liver is the site at which DES exerts its influence.

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CHANGES IN TYPE RATINGS OF HOLSTEIN COWS DUE TO AGE, SEASON, STAGE OF LACTATION, CLASSIFIER, AND YEAR [1]

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SUMMARY

Ratings of 233 Holstein cows, classified a total of 2,272 times from 1945 to 1957 by official classifiers, were coded to maintain equal intervals (1.0) between ratings. Least-squares analysis using dummy variables measured the effects of age with ratings grouped into Lactations 1, 2, 3, 4, 5, 6+; season, spring or fall; stage of lactation, parturition to 60 days, 61-240 days, and 241 days to next parturition; and classifier, 1 through 13. Linear year effects also were measured. Analysis on a within-cow basis removed possible animal bias. Constants, calculated as partial regression coefficients, represented the additive effect of each environmental factor independent of all others.

On a within-cow basis, coded over-all ratings increased steadily with age, with a range of 0.92 between Lactation 1 and 6+; spring ratings were higher than fall by 0.07**; early- and late-lactation scores were higher (0.13* and 0.17**) than mid-lactation. The range among classifiers was 1.38. Trends among subratings were similar except that feet and legs, and fore udder, showed smaller age and greater classifier effects; in late lactation, dairy character decreased (0.17**) and body capacity increased (0.27**). The linear decrease in over-all ratings was 0.03** per year, with a range of 0 to −0.06** for the subratings.

In establishing the true relationships between type and economically important characteristics in dairy cattle, it is necessary to establish a common basis for the comparison of animals with each other. Because of variation in many factors which may conceivably influence a single type value, such a value for a cow may not be an accurate indication of the cow's inherent characteristics. A single value adjusted for some of these outside influences is needed.

A previous investigation and review of the literature by the authors (7) revealed that several factors, environmental in character, systematically biased official type ratings. Though experts in their field, official classifiers were not able to avoid either transitory or consistent fluctuations in making their subjective evaluations of animals; often this variation has been described as human error. Real changes occurred in appearance of the animals with age and with stage of lactation. Classifications took place in different seasons and in different years. These five influences (classifier, age, stage of lactation, season, and year) could logically bias scores for over-all rating, and for the various subratings; they are hereafter referred to as environmental factors. Measurement and evaluation of these effects in an attempt to obtain more valid estimates of type seemed justified, and were the objectives of this investigation.

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Previous studies were hampered by difficulties encountered in the analysis of large amounts of data for a large number of major effects through use of desk calculators. The recent availability of an electronic computer (IBM 650) to this station has made possible more comprehensive investigations.

EXPERIMENTAL PROCEDURE

Data covering 13 yr. (1945 to 1957) were available from official type classifications of the Holstein-Friesian herd owned by the New Jersey Agricultural Experiment Station. During this time, all adult females were classified twice annually. A more complete description of the population and classification schedule has been published previously (7).

A total of 278 cows were classified 2,708 times for the over-all rating and each of the eight subratings. As a part of a special project from 1945 to 1949, three classifiers rated all animals simultaneously and independently (6); from 1950 to 1957 only one classifier at a time did the scoring. Ratings were coded as follows: Excellent, rated as 6; Very Good, as 5; Good Plus, as 4; Good, as 3; Fair, as 2; and Poor as 1. Intervals between scores were thus kept equal.

Lactation numbers were used as a measure of age and ratings grouped into Lactations 1, 2, 3, 4, 5, and 6 or higher (6+). Cows with ratings in the 6+ group completed an average of 6.82 lactations. Ratings were also grouped according to season (spring or fall), and three periods according to stage of lactation: (1) from day of parturition to 60 days, (2) 61 days to 240 days, and (3) 241 days to subsequent parturition. During the 13-yr. period, 13 classifiers rated the animals. Several came only twice, but one came 11 times; the average was 4.18 times.

Data representing every available animal classification were assembled. Obviously abnormal ratings were then excluded, such as many of those closely preceding or following abortions, and many terminal classifications prior to disposal. Since rather complete milk production and health records were kept, individual attention was given to borderline cases. Also excluded were animals which had been classified only once. There remained 2,272 classifications of 233 cows for a mean number of ratings per cow of 9.75.

A scheme of analysis was developed (4) combining the dummy variable and least-squares procedures, as described by Hazel (2) and Lucas (3), with the standard analysis of multiple covariance.

The nine type characteristics were numbered $1 \ldots h \ldots 9$; these included over-all rating, general appearance, dairy character, body capacity, mammary system, fore udder, rear udder, feet and legs, and rump. Environmental categories were numbered $1 \ldots i \ldots 6$. The categories, and the specific variables $1 \ldots j \ldots v$, were distributed in the following manner:

	Category *(i)*	Variable *(j)*
1.	Over-all	1
2.	Lactation number	1 to 6
3.	Season	1 to 2
4.	Stage of lactation	1 to 3
5.	Classifier	1 to 13
6.	Year	1

The animals were numbered 1 . . k . . 233, and the observations within each animal 1 . . l . . o. The linear model representing these effects, which were assumed to be additive, was as follows:

$$Y_{hijkl} = \mu_h + \sum_{ij}\sum b_{hij}X_{hij} + a_{hk} + e_{hijkl}$$

where Y_{hijkl} = the type score for characteristic h, at the lth observation on the kth animal under conditions described by the jth variables in the ith categories,

μ_h = the base value for characteristic h when all the X_{hij} are 0,

b_{hij} = the constant representing the effect on characteristic h of the jth variable in the ith category,

X_{hij} = the independent variable taking the value 1 or 0 for $ij = 11$ to 5 13 (dummy variables), or the coded value for year, a continuous variable, ($ij = 61$) ; the same for all values $h = 1$ to 9,

a_{hk} = the effect on characteristic h of the kth animal, and

e_{hijkl} = the error term including random error and any interactions among the main effects which may have existed, though none was assumed to have existed.

In the scheme of analysis used, one equation in each category based on dummy variables, except one, could not be considered independent. The simplest solution was to set one constant in all but one category equal to zero. The other constants in the category were then measured not as deviations from mean but as deviations from the variable which was set equal to zero. Calendar year was incorporated as a continuous variable and thus was not deleted; year was coded by subtracting 1900 from the actual year, e.g., 1948 − 1900 = 48. Selection of constants to set equal to zero may be quite arbitrary. Lactation 4 was selected as a point at which the dairy cow reaches maturity in size and production. Spring was selected over fall, and Period 2 over Periods 1 and 3. Classifier 3 was selected because he had classified the most times (11), with the most animal-classifications (539), and thus afforded the maximum number of within-animal and within-classification comparisons with other inspectors.

The term μ_h thus reflected the base value, or mean for type rating h for Lactation 4 ($ij = 24$), spring (31), Period 2 (42), and Classifier 3 (53). With Variable 61 a continuous variable, μ_h also reflected extrapolation back to 0, the coded value for year 1900.

The a_{hk} term, representing animal effects, was taken into consideration by the analysis of multiple covariance approach, in which three matrices of sums of squares and sums of products were set up: total, among cow, and within cow. Since the objective in this study was to eliminate the effect of genetic differences among cows on the constants, they were calculated from the within-cow matrix. When effects are determined by this procedure, and if the assumption that no real interactions exist is correct, each effect can be considered to be independent of, or adjusted for, all other effects.

RESULTS

Unadjusted means of the ratings grouped according to lactation number, season, stage of lactation, and classifier are presented (Table 1), along with the percentage of the total number of classifications in each category. With over-all ratings, and with most of the subratings, these mean scores increased with age; fall ratings were slightly lower than those in spring; marked changes occurred during the lactation; and classifier means varied considerably.

The least squares estimates of the effects of the five environmental factors (Table 2), calendar year being included as a continuous variable, were those obtained on a within-cow basis. The 1 and 5% levels of significance also have been presented.

It was apparent that animal effects very nearly had been randomly distributed. Their removal did not affect to any great extent the constants obtained. The total (not shown) and within-cow constants were very nearly alike. Furthermore, both sets of constants were similar to the simple arithmetic differences in the means, probably indicating a lack of any important interactions.

The statistical significance should be interpreted with caution. In the case of age, trends were probably more important than actual significance. Since Lactation 4 lay between 3 and 5, these lactations could have been an integral part of a trend without being significant, and such was usually the case. A somewhat comparable situation existed with stage of lactation, since the tendencies were usually for ratings to drop from Period 1 to 2 and rise again in 3. However, tests of significance of season effects were quite valid, because only one comparison (fall vs. spring) was made. Similarly, with the linear calendar-year term, the tests of significance validly indicated the presence of year effects. With classifier effects, observation of the mean values for Classifier 3 showed this person to be one of the stricter classifiers. Thus, the more lenient classifiers usually differed significantly. Had an inspector been selected whose ratings were close to the mean, his immediate neighbors very probably would not have differed significantly.

On the last line of Table 2 are the percentages of the within-cow sums of squares which could be attributed to the combined effects of the five environmental factors. These values ranged from 14 (fore udder) to 28% (feet and legs). As indicated in the mathematical model, the remaining within-cow variation consisted of that which was truly random and that which was due to interactions of any of the environmental factors with each other. No interactions between these variables and the animals themselves would be included, however.

The increase in ratings with age did not proceed in a linear manner, as is shown graphically for over-all rating in Figure 1. The upward trend observed from the simple means was more pronounced than that from plotting the within-cow constants, due to the animal effects on the former; most low-classifying animals were eliminated from the herd as young cows and thus reduced the means for the lower lactation numbers but not for the higher numbers. The constants were plotted by using the mean for Lactation 4 as the base. An asymptotic curve

TABLE 1

Means of type ratings falling into various environmental categories

Environmental category		Per cent of total	Type ratings								
			Over-all	General appearance	Dairy character	Body capacity	Mammary system	Fore udder	Rear udder	Feet and legs	Rump
Lactation	1	30	3.34	3.45	3.89	3.90	3.32	3.39	3.37	3.10	3.47
Lactation	2	24	3.57	3.67	4.21	4.06	3.48	3.36	3.59	3.18	3.79
Lactation	3	19	3.90	4.00	4.47	4.30	3.73	3.56	3.79	3.29	4.03
Lactation	4	11	4.04	4.08	4.65	4.56	3.72	3.56	3.76	3.18	4.06
Lactation	5	6	4.10	4.19	4.76	4.62	3.83	3.54	3.94	3.39	4.10
Lactation	6+	10	4.44	4.43	4.86	4.82	4.16	3.95	4.31	3.82	4.29
Season	S	50	3.77	3.86	4.38	4.28	3.64	3.49	3.75	3.30	3.82
Season	F	50	3.70	3.78	4.24	4.17	3.55	3.50	3.60	3.21	3.87
Stage	1	15	3.70	3.79	4.36	4.05	3.62	3.50	3.76	3.47	3.78
Stage	2	42	3.63	3.71	4.37	4.11	3.47	3.36	3.56	3.17	3.76
Stage	3	43	3.85	3.93	4.24	4.40	3.71	3.64	3.76	3.25	3.94
Classifier	1	6	3.82	3.95	4.21	4.43	3.58	3.58	3.45	2.93	3.86
Classifier	2	8	3.58	3.51	4.31	4.36	3.33	3.19	3.51	3.21	3.80
Classifier	3	24	3.55	3.68	4.07	4.02	3.36	3.29	3.39	2.69	3.61
Classifier	4	6	3.85	4.19	4.07	4.18	3.54	3.47	4.03	3.99	4.48
Classifier	5	7	3.99	4.00	4.33	4.11	4.14	4.04	4.09	3.69	3.86
Classifier	6	6	3.80	3.94	4.56	4.54	3.72	3.57	3.99	3.28	4.35
Classifier	7	3	4.20	4.22	4.36	4.51	3.95	3.87	3.91	3.84	4.13
Classifier	8	4	3.67	3.67	4.13	4.17	3.43	3.18	3.74	3.45	3.55
Classifier	9	15	3.74	3.84	4.81	4.16	3.78	3.62	3.73	3.26	3.73
Classifier	10	4	4.10	4.24	4.47	4.64	3.99	4.06	3.98	3.99	4.11
Classifier	11	5	3.70	3.72	4.25	4.25	3.52	3.44	3.64	3.39	3.79
Classifier	12	6	3.52	3.51	4.14	4.11	3.27	3.20	3.28	2.90	3.63
Classifier	13	6	3.55	3.54	4.22	4.26	3.49	3.42	3.65	3.44	3.41
Average			3.74	3.82	4.31	4.22	3.60	3.50	3.67	3.25	3.84

TABLE 2

Effects of five environmental factors measured on a within-cow basis and expressed as deviations from zero

Environmental category		Type ratings								
		Over-all	General appear-ance	Dairy character	Body capacity	Mammary system	Fore udder	Rear udder	Feet and legs	Rump
Lactation	1	−0.51**	−0.43**	−0.65**	−0.38**	−0.30**	+0.03	−0.37**	−0.06	−0.54**
Lactation	2	−0.29**	−0.24**	−0.30**	−0.25**	−0.14**	−0.02	−0.15**	−0.05	−0.25**
Lactation	3	−0.11*	−0.04	−0.13*	−0.12*	−0.00	+0.03	+0.02	+0.09	−0.01
Lactation	4	0	0	0	0	0	0	0	0	0
Lactation	5	+0.10	+0.12	+0.16*	+0.11	+0.11	−0.04	+0.15*	+0.17*	+0.05
Lactation	6+	+0.41**	+0.34**	+0.17**	+0.22**	+0.38**	+0.26**	+0.46**	+0.45**	+0.20**
Season	S	0	0	0	0	0	0	0	0	0
Season	F	−0.07**	−0.09**	−0.07*	−0.16**	−0.06*	+0.03	−0.14**	−0.03	+0.02
Stage	1	+0.13**	+0.11**	+0.04	−0.02	+0.24**	+0.21**	+0.31**	+0.34**	−0.06
Stage	2	0	0	0	0	0	0	0	0	0
Stage	3	+0.17**	+0.19**	−0.17**	+0.27**	+0.19**	+0.21**	+0.20**	+0.07*	+0.20**
Classifier	1	+0.19*	+0.20**	+0.10	+0.34**	+0.09	+0.14**	−0.03	+0.12	+0.30**
Classifier	2	+0.07	−0.14*	+0.27**	+0.38**	+0.07	+0.04	+0.18**	−0.64**	+0.04
Classifier	3	0	0	0	0	0	0	0	0	0
Classifier	4	+0.37**	+0.57**	−0.00	+0.16*	+0.37**	+0.54**	+0.74**	+1.46**	+0.84**
Classifier	5	+0.27**	+0.16**	+0.18**	−0.18**	+0.53**	+0.51**	+0.46**	+0.77**	+0.32**
Classifier	6	+0.27**	+0.27**	+0.48**	+0.53**	+0.48**	+0.58**	+0.64**	+0.71**	+0.58**
Classifier	7	+0.51**	+0.42**	+0.25**	+0.32**	+0.40**	+0.38**	+0.35**	+0.91**	+0.61**
Classifier	8	+0.12	+0.01	+0.06	+0.10	+0.02	−0.17*	+0.33**	+0.70**	+0.10
Classifier	9	+0.09*	+0.07	+0.66**	+0.03	+0.31**	+0.23**	+0.23**	+0.51**	+0.08
Classifier	10	+0.25**	+0.29**	+0.25**	+0.23**	+0.24**	+0.40**	+0.21*	+0.97**	+0.49**
Classifier	11	+0.68**	+0.63**	+0.32**	+0.32**	+0.79**	+0.90**	+0.90**	+1.54**	+0.91**
Classifier	12	−0.68**	−0.75**	−0.21	−0.19	−0.90**	−1.05**	−0.98**	−1.31**	−0.82**
Classifier	13	−0.70**	−0.77**	−0.22*	−0.13	−0.70**	−0.80**	−0.67**	−0.84**	−1.01**
Year		−0.03**	−0.03**	−0.00	+0.00	−0.06**	−0.05**	−0.06**	−0.06**	−0.05**
Reduction (%)		22	20	22	17	18	14	21	28	23

** Significant at the 1% level.
* Significant at the 5% level.

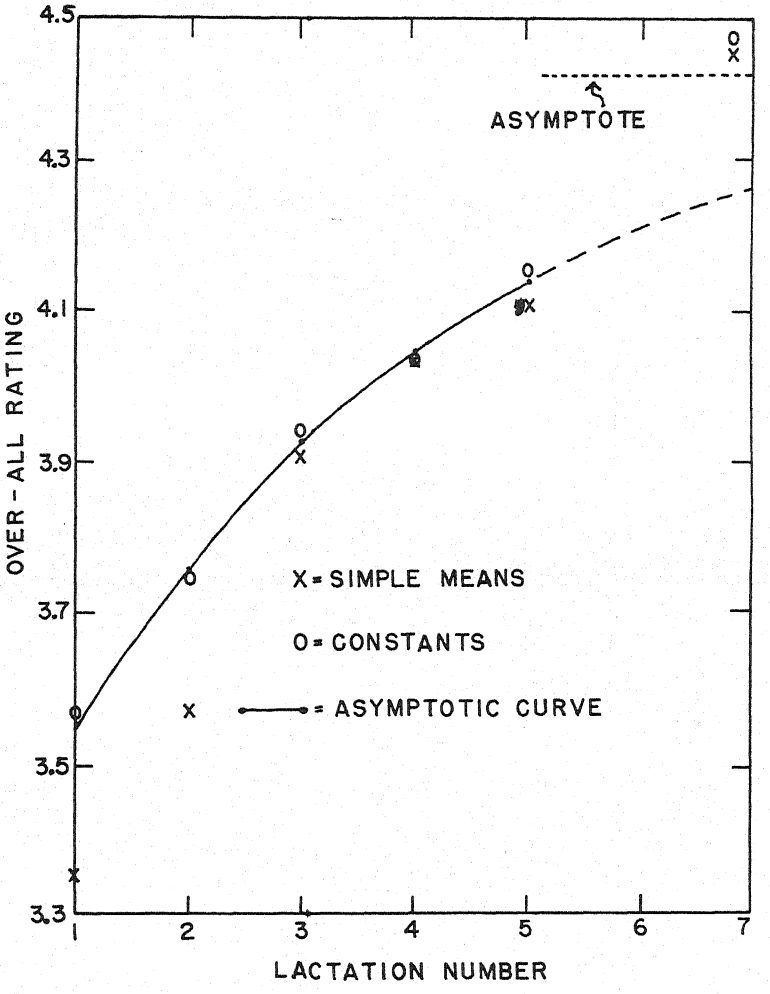


FIG. 1. An asymptotic curve representing the effects of age on over-all rating.

was calculated using the simple procedure described by Patterson (5); in this type of curve, the line approaches a maximum (asymptote) at an ever-decreasing rate. This might be expected in these data unless some other effect entered the picture. As possibilities, old age might have a tendency to reduce the rating, or greater leniency on the part of the classifier might tend to increase the rating more than otherwise would be expected. To evaluate the net effect of these possible influences, the asymptotic curve was fitted to the constants for Lactations 1 to 5, and extrapolated to the value for the Lactations 6+ data (mean 6.82 lactations) for comparison with the observed value.

The calculated curve fitted the plotted constants exceptionally well (Figure 1). The standard error of estimate of the within-cow constants was 0.0063 after fitting the curve. The closeness of fit also may be shown by the forecasting efficiency (1) of 97.4% and the correlation coefficient of .9997, though neither

of these measures is free of the influence of selection of the fixed independent variable.

With the very close fit of the curve to the data, an extrapolated value would be subject to relatively small error. For Lactation No. 6.82, the calculated expected value was 4.25 ± 0.006, which was markedly below the simple mean (4.44) and the comparable constant (4.45). These latter values also were slightly higher than the asymptote or theoretical maximum for an infinite number of lactations (4.41).

DISCUSSION

When the confounding of the other environmental factors was removed from age, it was shown that a definite and statistically significant age effect remained. Where the range between grades was five score-card points, as with good to good plus, or good plus to very good, etc., over-all ratings of first-calf heifers differed from mature (6+) ratings by 4.6 points (0.92×5). First-lactation animals could be expected to be raised almost one whole rating, on the average, if they remained in the herd long enough. Similar trends existed with all subratings except fore udder and feet and legs. Ratings for these two traits for animals in the sixth or later lactations were considerably higher than in Lactations 1 to 5, however.

The question of whether cows actually improved in type or whether they improved only in the eyes of the classifier must remain academic. It has been an admitted and probably desired policy of inspectors to be stricter with younger animals, and inspectors also have probably been more sympathetic toward the older, proven animals. It did not seem logical that feet and legs, or rumps, were actually improved by stresses of aging. It was also indicated, by the marked increase noted from Lactation 5 to 6+ in most type ratings, that the inspector's allowance for the very old cows was greater than it should have been. This was demonstrated by the differences between the observed and constant values and the expected value on the asymptotic curve (Figure 1).

Cows were rated higher after the winter feeding season than after the summer feeding season. The combined weight of more desirable mammary systems, body capacity, dairy character, and general appearance resulted in a higher (0.07**) over-all rating. The rather striking influence of season on rear udders was apparently great enough to affect mammary systems as a whole. It would seem that breeders striving for high average scores should classify in the spring, if their management and climatic conditions are similar to those of the population studied.

Over-all ratings were higher in late-lactation than in mid-lactation. Significantly lower dairy character ratings were more than offset by large increases in body capacity scores, along with smaller increases in all other subratings. Pregnancy and weight gains after the peak of lactation were probably responsible for increases in body capacity and general appearance; moreover, defects in mammary system were probably not as apparent in late-lactation as in mid-lactation. Shortly after freshening, animals were also consistently scored high. Though no significant changes occurred in dairy character, body capacity, and

rump, decreases in scores from early- to mid-lactation ranged from 0.11** (general appearance) to 0.34** (feet and legs) for the remaining ratings.

Removal of confounding environmental effects slightly accentuated differences among classifiers. Although the range among averages for over-all ratings was 0.68 (between Classifiers 7 and 12), the range for the calculated constants was 1.38 (for Classifiers 11 and 13). Differences were even more evident among the minor subratings, fore and rear udder, rump, and feet and legs.

During the last 5 yr. of the study, ratings were made by Classifiers 2, 3, 4, 6, and 11. Ranges among their means amounted to only 0.25 and among the constants only 0.68. With the advent of full-time professional classifiers, it is evident that some progress has been made in the establishment of a more uniform standard for classification.

Classifiers whose over-all ratings averaged higher or lower than Classifier 3 did not necessarily score the subratings likewise, although such was usually the case. The average deviation of over-all ratings of Classifier 2 was +0.07, but he was relatively strict on feet and legs (−0.64**) and easy (+0.38**) on body capacity.

Opposing the increase in scores with age were the decreases in the score by progressing calendar years. Negative in eight of nine ratings, and highly significantly so in seven of nine, these linear partial regression coefficients demonstrated a marked trend over the 13-yr. period. The underlying reasons for these effects were not clear, but it may be postulated that classification policies became stricter. Staff members who were associated with the herd for the whole period believed that the type of the herd had improved; however, the average score had not. Raising of standards by classifiers could have been the explanation. If true, of course, this by no means could be considered an undesirable trend. Difficult if not impossible to prove, similar trends were in evidence nowhere else in the literature.

With 22% of the within-cow variation among over-all ratings due to the five environmental factors, it would seem important to consider these factors in studies of type ratings and subratings. They should be considered to be a source of potential bias until proven otherwise. Gross differences among means should point out the major trends, if these trends exist in dairy cattle in general to the degree to which they existed in the Holstein population investigated.

If the decrease in the range among classifier means is indicative, it is possible that a more consistent standard has been reached by the classifiers in the recent years. It is probable, however, that the very old cows (6+) are still being given an over-allowance for age. Classifiers raised scores more in this period than expected from their previous systematic rate. Additional improvement would seem attainable in scoring dairy character and body capacity during the lactation. Since the stage of lactation of each animal is known to the classifier, he undoubtedly already adjusts his scores for these characteristics to some degree. If the true inherent type of the animal is best represented by all of its scores, however, the classifier is still being too strict on dairy character in late-lactation, and too lenient on body capacity during the same period.

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EFFECT OF A SIMPLE HIGH-FIBER FEED ON DAIRY CALVES[1]

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SUMMARY

Thirteen pairs of calves including Jersey, Guernsey, and Holstein breeds were used to evaluate the effect of complexity and fiber content of concentrate mixtures for young calves. A simple high-fiber mixture containing 13.9% fiber was consumed as readily as was a complex low-fiber feed containing 6% fiber. No differences in tendency to consume hay were observed. All calves gained considerably more than the expected gain as computed by use of the Ragsdale standard (6), and no differences in rate of growth or general health were apparent.

Rumen functions develop considerably earlier in the life of the calf than previously was thought. Fermentation was observed to be well established in the stomach of calves 20 to 30 days old (4). Close observation has shown that rumination occurred in calves as young as 2 wk. (8). It also has been shown by the Ohio workers (2) that a high roughage ration is practical for young calves. Under conditions of this experiment, a complex calf meal was not necessary, but whole milk was fed through 7 wk. of age.

Concentrate feeds with some characteristics of mature ruminant feeds also appeared to be practical. Whitaker *et al.* (9) found that addition of alfalfa meal and/or corn cobs and shucks to bring the fiber content of calf meal to 13% did not affect its value as a feed for young calves. The original concentrate feed, however, was quite complex.

A very simple ration containing corn, oats, soybean oil meal, molasses, and minerals was reported by Murley *et al.* (5) as being fully as satisfactory as was a complex starter for young calves. This ration, however, was not particularly high in fiber.

Hollon *et al.* (3) observed that hydraulic process cottonseed meal had toxic effects on young calves; whereas, screw process cottonseed meal was well tolerated. Whitaker *et al.* (9) used cottonseed meal which had been degossypolized, and Murley *et al.* (5) used soybean oil meal. Neither of these meals had toxic effects. The protein source most readily available in the Southeast, solvent process cottonseed meal, has not received attention as a constituent of simple calf feeds.

If young calves can use a concentrate which is simple and also is high in fiber, only one ration should be needed for all ages of dairy animals. In the present study an ordinary high-fiber, simple cow herd concentrate mixture containing solvent process cottonseed meal was compared to a low-fiber complex meal as feed for young calves.

EXPERIMENTAL PROCEDURE

Sixteen calves born during the fall of 1957 and spring of 1958, and ten born during the fall of 1958 and spring of 1959, were paired when four days of age. The pairs were balanced with respect to breed, sex, body weight, and height at

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withers. One member of each pair was offered a complex calf meal containing 300 lb. ground shelled corn, 250 lb. ground oats, 100 lb. wheat bran, 130 lb. extraction process cottonseed meal, 41% protein, 100 lb. nonfat dry milk solids, 8.5 lb. common salt, and 17 lb. steamed bone meal. This mixture contained 18% crude protein and 6% crude fiber as determined by standard methods (1).

Pair-mates received a concentrate mixture identical with the one used for the station milking herd. It contained 600 lb. ground snapped corn, 400 lb. citrus pulp, 200 lb. extraction-process cottonseed meal, 41% protein, 14 lb. common salt, and 28 lb. steamed bone meal. This mixture was 17.8% protein and 13.9% fiber.

All subjects were removed from their dams at birth. They were confined in individual pens which were bedded with wood shavings. Good-quality sun-cured Pangolagrass hay was offered free choice.

The milk ration offered in two equal portions daily was fed at the rate of 9% of body weight. Nipple pails were used during the first 3 wk. and thereafter milk was fed from open pails. Fresh colostrum, which was a mixture of the first four days' milk from Jersey, Guernsey, and Holstein cows, was fed during the first four days. A mixture of half colostrum and half skimmilk was fed between the ages of five and 21 days, inclusive. Thereafter, skimmilk was

TABLE 1

Effect of fiber contents of concentrates on growth and feed consumption by calves to 60 days of age

Calf No.	Breed and sex	Body weight		Height		Feed consumed	
		Initial	Gain	Initial	Gain	Concentrate	Hay
Control[a]		(lb.)		(cm.)		(lb.)	
1	HF	87	99	72	10	112	7
3	HM	102	92	77	9	60	28
5	JM	85	75	74	12	66	7
7	GM	81	63	79	8	55	10
9	JM	56	42	68	7	67	15
11	GF	78	62	75	10	52	6
13	JM	60	60	68	11	52	12
15	JF	53	49	64	10	46	13
17	JF	52	45	66	9	37	12
19	GM	79	58	75	8	56	13
21	JF	55	47	68	7	35	22
23	JM	60	37	69	7	46	13
25	HF	97	50	79	12	52	7
Average		73	60	72	9	57	13
High fiber[b]							
2	HF	84	85	70	11	91	5
4	HM	102	95	81	9	55	32
6	JM	85	80	73	10	84	6
8	GM	81	53	75	11	74	11
10	JM	57	46	66	9	48	9
12	GF	78	50	74	7	39	5
14	JM	60	52	66	8	60	15
16	JF	55	57	66	9	74	14
18	JF	55	55	67	8	54	10
20	GM	75	65	74	10	88	4
22	JF	53	55	67	10	42	3
24	JM	60	50	69	8	64	10
26	HF	97	83	77	10	62	35
Average		72	64	71	9	64	12

[a] The control ration contained 6% of fiber.
[b] The experimental ration contained 13.9% of fiber.

fed. The skimmilk was reconstituted at the rate of 1 lb. of air-dry solids per gallon. Vitamin A, 15,000 I.U. per head daily, was dispersed in the milk.

All concentrates and hay in the feeders were removed once daily, the consumption rate recorded, and a fresh offering supplied. Weekly records were maintained on body weights and heights at withers; observations were made three times daily to determine whether differences occurred in general appearance and health. The records were terminated when the calves reached 60 days of age, and all data were analyzed statistically, using the technique described by Snedecor (7) for paired data.

RESULTS AND DISCUSSION

No scouring or other abnormalities were observed, all calves appeared vigorous, and no differences in general appearance and health were discernible. Two calves in the high-fiber group consistently selected the fine particles, leaving, for the most part, corn-cob particles in the feed box.

Calves in both groups consumed both concentrates and hay readily (Table 1). Growth was above normal (6) in all calves, and type of concentrate feed did not have any effect which could be considered significant. These data confirm the results of Whitaker *et al.* (9) and Murley *et al.* (5). They show further that the concentrate feed for young calves may be both simple and high in fiber.

The effect of solvent process cottonseed meal was not planned as a part of this experiment. It was included in both rations and, though no negative control was maintained for comparison, it appeared to have no ill effect. In so far as complexity and fiber content are concerned, there appears to be no reason to supply special concentrate mixtures for young calves under the conditions of this experiment.

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TECHNICAL NOTES

TEMPERATURE INCREASE DUE TO HOMOGENIZATION

The approximate temperature increase in °F. in a product due to homogenization may be estimated by dividing the homogenizing pressure (p.s.i.) by 300. More accurate estimates are obtained by dividing the homogenizing pressure by factors specific for a given product.

Many teachers and technologists refer to the temperature rise in the product caused by homogenization in terms of their casual observations, without regard to the variation with pressure.

It is readily seen that the temperature rise must be the result of energy input dissipated as heat. This heat is the equivalent of the hydraulic horsepower input. An equation for temperature rise can be derived as follows:

B.t.u.'s gained by product/hour = weight of product/hour × temperature change (°F.) × specific heat

$$\text{B.t.u.'s} = W \Delta t s \qquad (1)$$

B.t.u.'s expended by homogenizer = B.t.u. equivalent to horsepower hour × hydraulic horsepower = 2,545 h.p. (2)

$$\text{Hydraulic horsepower} = \frac{\text{weight of product/hour} \times \text{feet head}}{33{,}000 \text{ ft. lb/min} \times 60 \text{ min.}} = \frac{WH}{1{,}980{,}000} \qquad (3)$$

Equation (1) must equal (2)

$$W \Delta t s = 2{,}545 \text{ h.p.}$$

Now substitute (3) for h.p.

$$W \Delta t s = \frac{2{,}545\, WH}{1{,}980{,}000} = \frac{WH}{778}$$

Solve for Δt:

$$\Delta t = \frac{WH}{778\, Ws} = \frac{H}{778\, s} \qquad (4)$$

To use the homogenizer gauge pressure (p) directly, convert feet of head (H) to pounds per square inch (p.s.i.) pressure (p):

$H = \frac{p}{d}$, where d = weight of product contained in a 1-in.-square column, 1 ft. high.

$= \frac{p}{.425}$ for water (at 150° F. homogenizing temperature).

Then $\Delta t = \frac{H}{778\, s} = \frac{p}{778\, s\, .425}$

and since specific heat, $s = 1$ for water

$$\Delta t = \frac{p}{331} \text{ for water}$$

Table 1 gives dividers for other products where more accurate estimates are desired.

The equation shows that the flow rate and type of homogenizer have no effect on the temperature rise.

TABLE 1

Dividers for converting pressure (p.s.i.) to ° F. temperature rise

Product	Density factor (150° F.) d	Specific heat[a] s	Divider $778 \cdot ds$
Water	.425	1.00	331
Milk	.440	.93	318
12% Cream	.435	.90	305
20% Cream	.432	.88	296
Evaporated milk	.454	.92	325
Ice cream mix	.464	.80	289

[a] Values taken from tables and/or extrapolated from curves given in Chapter 1, Arthur W. Farrall, *Dairy Engineering*, 2nd ed. John Wiley & Sons, Inc., New York. 1953.

TABLE 2

Comparison of temperature rise observed on actual trials with computed value-water

Homogenizing pressure	Temperature rise, °F. 400 gal/hr[a]	4,000 gal/hr[a]	Computed
(p.s.i.)			
600	....	2.0	1.8
800	2.5	2.5	2.4
1,000	3.4	3.2	3.0
1,200	4.0	3.6	3.6
1,400	4.5	4.2	4.2
1,600	5.0	5.0	4.9
1,800	5.6	5.3	5.4
2,000	6.3	6.3	6.1
2,200	....	6.8	6.7

[a] Average of three to five measurements.

To check the actual temperature rise at various homogenizer pressures against the approximate computed values, a 400–g.p.h. homogenizer and a 4,000–g.p.h. homogenizer were set up in the laboratory, with copper-constantan themocouples attached at the intake and discharge connections. Water was pumped through the homogenizers and subjected to pressures ranging from 800 to 2,200 p.s.i. The temperatures of the water at the intake and at the discharge were measured with the thermocouples and a 12–pt. Brown Electronik potentiometer. The results given in Table 2 show very close agreement with the computed values.

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SOME CHARACTERISTICS OF THE GLYCO-MACROPEPTIDE OF CASEIN—A PRODUCT OF THE PRIMARY RENNIN ACTION [1]

The primary action of rennin on casein and the concurrent release of nonproteolytic NPN have been the subject of recent investigations. Recently, Wake (5) showed that κ-casein, the calcium-insensitive protective colloid of the α-casein complex, was the substrate for the primary action of rennin. Alais (1) isolated two groups of peptides from the NPN fraction. One group, soluble in 2% TCA, was found to be electrophoretically heterogeneous and contained a major component which constituted 90% of the total 12% TCA-soluble constituents. The electrophoretic mobility of this component was reported at 7 to 8 cm.^{2} v.$^{-1}$ sec.$^{-1}$ × 10^{-5} in veronal buffer at pH 8.6. Nitschmann *et al.* (4) found this component, ranging in molecular weight between 6,000 and 8,000, to be rich in carbohydrate and, consequently, the term glyco-macropeptide was used to describe the peptide.

The objective of our studies has been to elucidate the composition and physical characteristics of this electrophoretically homogeneous glyco-macropeptide.

Casein was prepared by isoelectric precipitation from fresh skimmilk, reprecipitated twice from alkaline solution, and subsequently washed. The wet casein was made up to 3% concentration in dilute NaOH, adjusted to pH 7.0 with 10% HCl, and dialyzed against distilled water for 12 hr. at 4° C. Crystalline rennin (Sigma Chemical Co.) was added to the sodium caseinate sol and the reaction allowed to proceed for approximately 1 hr. at 25° C. At this point, the ascending electrophoretic pattern showed a split in the α-casein peak.

The enzyme-treated sol was adjusted to pH 4.6 to precipitate the casein. The clear supernatant was dialyzed to remove the HCl and concentrated by pervaporation. A 20% solution of freshly prepared TCA was added slowly to the concentrated solution with constant stirring until a final concentration of 12% TCA was reached. The precipitated peptides were removed by centrifugation (≅ 1,000 × G for 30 min.). The clear supernatant was adjusted to pH 7 with NaOH solution and dialyzed to remove TCA. The TCA extraction step was repeated two times before lyophilizing the glyco-macropeptide solution.

The electrophoretic characteristics of the macropeptide were observed in the following buffers: acetate, pH 4.2 and 4.7; phosphate, pH 5.9 and 7.4; veronal, pH 8.6 (pH values at 23° C., Γ/2 = 0.1). All of the solutions for electrophoretic analysis contained 1.5% macropeptide. The electrophoretic patterns are shown in Figure 1. The peptide appears to dissociate at low values of pH as indicated by the lack of homogeneity and flatness of the migrating boundary. The peptide migrates as a single, homogeneous component in buffers of higher pH values. Mobility values are presented in Table 1. An extrapolation of the descending mobility values to zero mobility gives an I_P (pH of no electrophoretic migration) value of approximately pH 2.8.

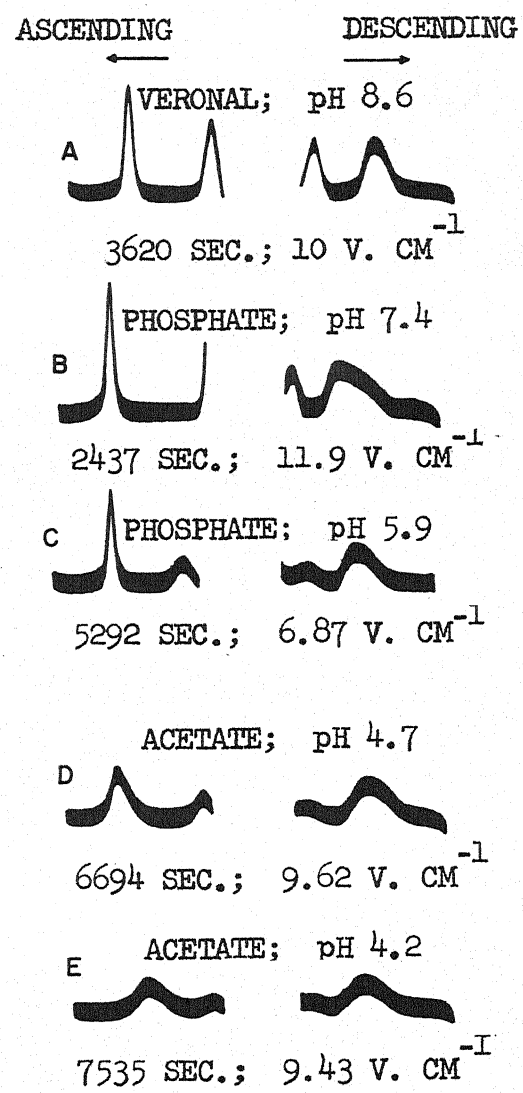


FIG. 1. The electrophoretic patterns of the glyco-macropeptide in various buffers. Concentration, 1.5%.

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TABLE 1
Electrophoretic mobility of the glyco-macropeptide in various buffers

Buffer[a] (Γ/2 = 0.1)		Electrophoretic mobility[b] ($\mu = \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$) Ascending pattern	Descending pattern
Veronal pH	8.6	6.18	4.62
Phosphate pH	7.4	9.19	5.46
Phosphate pH	5.9	5.97	4.75
Acetate pH	4.7	3.46	2.64
Acetate pH	4.2	2.54	2.09

[a] pH measured at 23° C.
[b] Measured from initial boundary.

Sedimentation analyses were performed at 28° C. in a Spinco Model E Ultracentrifuge, with 1, 2, and 3% solutions of glyco-macropeptide in veronal buffer, pH 8.6, Γ/2 = 0.1. The observed sedimentation constants were corrected in the usual manner and plotted. The $S_{20,w}$ values were extrapolated to zero concentration ($S^{c=o}_{20,w}$). This value was used in the calculation of the apparent molecular weight of the macropeptide. The sedimentation diagrams for the 3% solution and the plotted $S_{20,w}$ values for the 1, 2, and 3% solutions are shown in Figures 2 and 3, respectively.

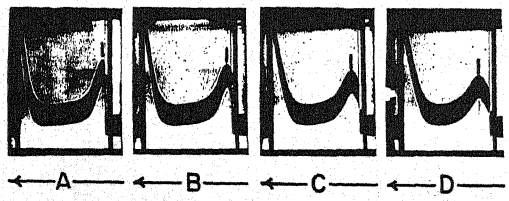


FIG. 2. The ultracentrifugal patterns of the glyco-macropeptide in the Spinco Model E Ultracentrifuge at 3% concentration in veronal buffer at pH 8.6, Γ/2 = 0.1.

The diffusion characteristic was observed at 20° C. in a Perkin-Elmer 38-A Electrophoresis Apparatus equipped with a cylindrical lens. The quantities $A^2/4\pi H^2$, where A and H are the area and height of the diffusion peak, were plotted as a function of t, the measured elapsed time. The slope of this plot represents the diffusion constant (7.5×10^{-7}). A partial specific volume (V) value of 0.68 was determined by means of the pycnometric method. Values determined for 1 and 2% solutions of macropeptide in veronal buffer at 20° C. were identical. Where required, relative viscosity values were determined by means of an Oswald viscometer. Density and viscosity values for water were taken from standard tables. The weight average molecular weight of the glyco-macropeptide, calculated from the sedimentation-diffusion method, was 15,263.

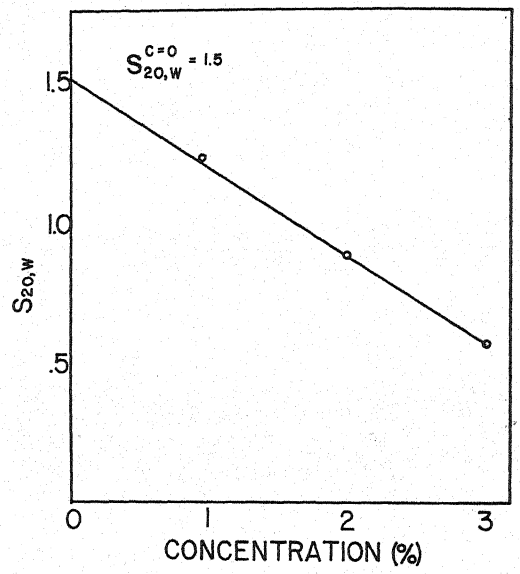


FIG. 3. $S_{20,w}$ plots for 1, 2, and 3% solutions of the glyco-macropeptide ($S^{c=o}_{20,w} = 1.5$).

Analyses for the various constituents of the glyco-macropeptide are listed in Table 2 and are compared with similar data reported previously by Nitschmann *et al.* (4) and Alais (1). Nitrogen, phosphorus, and neuraminic acid values were in good agreement. However, values for hexose and, to a lesser extent, glucosamine were lower in our glyco-macropeptide preparation. Nitschmann analyzed for the carbohydrate components by separation on chromatographic columns, whereas the techniques employed in this study are according to the scheme as outlined by Glick (2), which is capable of measuring individual protein-bound carbohydrates within a mixture. All of the standards were chemically pure, except for neuraminic acid which was obtained by using a standard sample of orosomucoid from hu-

TABLE 2
Chemical analyses of the glyco-macropeptide

Analysis	Fraction per cent: Reported values	Values from this study
Nitrogen	11.4[a]	11.7
Ash	....	6.0
Phosphorus	0.57[b]	0.63
Carbohydrate		
Hexose (galactose)	15.2[a,c]	5.1
Glucosamine	4.3[a]	2.3
Neuraminic Acid	11.4[a]	11.3
Total carbohydrate	30.9	18.7

[a] Hs. Nitschmann *et al.* (4).
[b] C. Alais (1).
[c] Recently, Nitschmann (3) obtained a hexose value of 5.9% for a more homogeneous preparation of the glyco-macropeptide.

man serum as a secondary standard (11.2% neuraminic acid).

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FORMATION AND REMOVAL OF AN IRIDESCENT DISCOLORATION IN CLEANED-IN-PLACE PIPELINES

In the course of some studies on in-place cleaning, Kaufmann *et al.* (2) reported the appearance of an iridescent discoloration on the interior of 18-8 stainless steel pipes after cleaning with an alkaline cleaning compound containing a nonionic wetting agent. Since this type of film has not been reported previously in the literature, this work was undertaken to determine some conditions of operation which would (a) cause the discoloration and (b) remove the discoloration.

Film build-up. The permanent CIP line used in this study consisted essentially of four sections of stainless steel pipe, each about 10 ft. long. Two sections were 500-grit finish, one was 300, and one was 180-grit finish. During the period of film deposition, the line was used for hot milk.

Immediately after use, the line was flushed with tap water at 90° F. and washed, using nonionic alkaline detergent "D" at the minimum recommended level. After flushing with tap water at 90° F. to remove the detergent solution, the line was chlorinated for 1-min., using 200 p.p.m. of chlorine. During soil build-up, circulation cleaning was carried out at 120° F. for 5 min. at a velocity of 4.8 ft. per second. Alkaline cleaner "D" contained polyphosphates, metasilicate, soda ash, and 2% nonionic wetting agent. The water hardness was not determined per se. Since the same supply was used in all studies, this factor remained a constant. Soiling with hot milk and cleaning as described was carried out for a period of 80 days. No acid cleaner was used during this period.

Film removal. Since film deposition occurred when cleaning was carried out at a low temperature and with minimum recirculation time, the first experimental cleaning trial was undertaken to study the effect of temperature on film removal. In the first trial, the temperature of detergent solution "D" was raised to 145° F. and recirculation carried on for 15 min.; the cleaning cycle was repeated 50 times. In the second trial, the temperature was raised to 160° F. and recirculation time increased to 30 min., since previous studies (2) indicated that a temperature of 160° F. for 120 min. removed the film. The results obtained in Trials I and II are given in Table 1.

In subsequent experimental cleaning trials, the CIP system was dismantled, to provide four individual pipe sections for study. Each section was cleaned as a separate unit on a CIP basis. This provided constant temperature control of the detergent solutions and constant recirculation velocity in each trial. With these factors held constant, it was possible to study the efficiency of various commercial detergents in removing iridescent film which had been permitted to build up. Each trial was carried out on the same section of pipe. When a procedure was found which removed the film, another soiled section of pipe was used to repeat the study, to eliminate interaction effects such as softening of the film by one process and removal by another.

In Trial III, anionic alkali No. 1 was used at the minimum level recommended by the manufacturer. This formulation differed essentially from the nonionic cleaner "D" used to produce the soil, in that it contained 8% anionic wetting agent and 6% less metasilicate. This solution was recirculated for 30 min. at 160° F. in Trial III. An alkaline bottle-washing compound was tested in Trial IV; this material also contained 8% anionic wetting agent, with a high level of alkalinity; it was recirculated for 30 min. at a temperature of

TABLE 1

Effect of various commercial cleaning compounds on the removal of iridescent discoloration in stainless steel pipes

Trial No.	CIP cleaning procedure			Description of pipe interior following treatment
	Temp.	Time	Detergent used	
	(°F.)	(min.)		
I[a]	145	15	Nonionic alkali D	Rainbow discoloration intense. Some black layer but no fatty deposits
II	160	30	Nonionic alkali D	Rainbow discoloration intense. No black spotting
III	160	30	Anionic alkali No. 1	Rainbow discoloration
IV	160	30	Anionic alkali No. 2	Rainbow discoloration
V	160	20	Organic acid	Rainbow discoloration
VI	140	20	Chlorinated alkali "A"	Some discoloration removed in less intense areas
VII	140	20	Chlorinated alkali "B"	No rainbow. Clean

[a] This trial was repeated 50 times.

160° F. An organic acid detergent at a level of 1 qt. per 30 gal. of water was recirculated for 20 min. at 160° F. in Trial V and two chlorinated alkaline cleaning compounds were tested in Trials VI and VII. Chlorinated alkali "A" was employed at the recommended level of 15 oz. per gallon of solution; it contained sodium hypochlorite, sodium metasilicate, and sodium phosphate as active ingredients. Chlorinated alkali "B" was used at a level of 8 oz. per 30 gal. (one-half the recommended level); on a dry basis the formulation of "B" contained the same percentage of sodium hypochlorite as "A" but considerably less metasilicate and wetting agent. Recirculation cleaning was carried out in both of these trials at 140° F. for 20 min. The results of all trials are summarized in Table 1.

Film deposition. Nonionic alkali "D" showed some evidence of film formation after seven days of use. At this time some slight rainbow discoloration and light brownish blotches were visible. After 28 days of use, an iridescent film completely covered the interior of all pipes. The continued use of detergent "D" in the cleaning operation at 120° F. for 80 days resulted in the formation of a thick, black, fatty layer which could be loosened by mild rubbing; this deposit was scattered throughout all pipes. In the cleaner areas (no black layer) some rainbow discoloration was noted. It is apparent from this that detergent "D" at 120° F. permitted formation and build-up of the film under the conditions described. This is in marked contrast to the anionic alkali No. 1, which did not permit film formation under identical conditions of use (1). It is interesting to note that very, very little iridescent film was observed in the CIP system after 1 yr. when nonionic alkali "D" was used at 145° F. rather than at 120° F. The importance of temperature in the formation of this film is indicated. It is apparent from the data (Trial I) that raising the temperature to 145° F. after the iridescent film had formed failed to eliminate it. The fatty residue was removed at the higher temperature, but the black layer and intense iridescent film remained clearly visible even after 50 trials.

Film removal. Although temperature played a role in film deposition, increasing the temperature of nonionic detergent "D" from 120 to 160° F. did not remove the discolored areas (Table 1, Trials I and II). Under the latter condition, the dark black stain was removed, but the intense iridescent areas remained clearly visible on the dry surface. Although anionic alkali No. 1 did not permit the iridescent film to be formed at 120° F., it could not remove the film at 160° F. (Table 1, Trial III).

Anionic alkali No. 2, used in Trial IV, was not effective in removing the film, although the active alkalinity of this formulation was considerably higher than that of anionic alkali No. 1. Alkalinity per se apparently does not play a major role in the removal of this film.

The results obtained in Trial V indicate that the commercial organic acid cleaner did not remove the intense discoloration after recirculation at 160° F. for 20 min. The question as to whether weekly acid treatment as is recommended in routine plant operation would have removed the film formed in seven days can not be answered with assurance at this time. From the foregoing it is evident that the removal of this film, once it is present, is not a simple problem with certain alkaline or acid detergents. From this it is apparent that preventative rather than removal techniques are to be emphasized.

Both chlorinated alkalies used in Trials VI and VII exerted some action on the film, but the efficiency of removal varied considerably. Detergent "A," at the recommended level, removed some of the film in the lighter areas after circulation cleaning at 140° F. for 20 min.; detergent "B," however, at one-half the recommended level, removed the film com-

pletely under the same test conditions. These trials were both repeated using two pipes showing discoloration. Results similar to the above were obtained. The sodium hypochlorite concentration is the same in both detergent formulations. Since the quantity of detergent "B" used was about one-half that of "A," it follows that the actual level of chlorine varied in the detergent solutions. It is also important to note that efficient film removal was observed at the lower concentration of chlorine. It has been suggested that the low metasilicate content in detergent "B" might permit greater detergent action, owing to a more favorable pH for chlorine activity (1).

A cleaning procedure is described whereby an iridescent film was built up in a stainless steel pipeline by using a selected commercially available nonionic detergent at 120° F. Increasing the temperature of the nonionic and anionic cleaner to 160° F. did not remove the film after it had formed. A highly alkaline bottle-washing compound and an organic acid cleaner failed to remove the film. Chlorinated alkali detergents varied considerably in their ability to remove the iridescent film.

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ADDENDUM

Since the completion of this study, a strong iridescent discoloration was observed on the glassware used in our laboratories. Replacing the commercial detergent routinely used with chlorinated alkali "B" removed the rainbow film on all test tubes and Petri plates.

PREPARATION OF A STANDARD FOR CITRIC ACID ANALYSIS

Considerable difficulty has been experienced with crystalline citric acid as a basis for preparation of a standard solution. Both the anhydrous and monohydrate forms of the acid are unstable at room temperature and humidity, with the result that the water content can vary between 0 and 2.5 *M/M*. Although the anhydrous acid is stable in a desiccator, it is difficult to obtain it by drying the monohydrate, because crystals of citric acid tend to melt at temperatures above 75° C. (4). Consequently, we have studied trisodium citrate as a standard material.

The composition of trisodium citrate dihydrate was verified by analyzing two lots (reagent grade) obtained from different manufacturers. The salt was of high purity and was probably obtained by recrystallization under controlled conditions of temperature and pressure (2). Sample A was taken from a freshly opened bottle, whereas the bottle containing Sample B had been opened for at least 1 yr. No preliminary drying was given either sample before analysis. Carbon was determined by dry combustion (1), water was estimated by heating to 150° C. overnight (4), and sodium was measured by flame photometry (5). The following results were obtained:

	CARBON (Theor.: 24.50)		WATER (Theor.: 12.25)		SODIUM (Theor.: 23.46)	
	A	B	A	B	A	B
Found, % by wt.:	24.28	24.36	12.36	12.19	22.70	22.95

All of these results fell within the experimental error of the various techniques.

Although this study indicated that trisodium citrate dihydrate was stable at room temperature and humidity, its stability was further studied by storing for three-day intervals under various extreme conditions. Exposure to dry 80° C. air caused a small loss in weight (0.041%). At room temperature, storage in the desiccator resulted in a negligible loss of weight (0.003%), whereas holding at 100% relative humidity caused a gain in weight of 3.65%. It is, therefore, apparent that trisodium citrate dihydrate is quite stable under normal laboratory conditions.

In a previous publication (3), a stock solution containing 50 mg. of anhydrous citric acid per milliliter was recommended, from which suitable standards could be prepared. On the basis of the above study of sodium citrate, the following preparation is now recommended: 76.4712 g. of trisodium citrate dihydrate (reagent grade) is made up to one liter with water. This solution is 0.260 *M* and, therefore, compares with one containing 50 mg. of anhydrous citric acid per milliliter. The solution is stored at 0° C. Fresh dilutions

of this stock solution are prepared each month and are also stored at 0° C.

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THE METABOLISM OF SERINE-3-C^{14} BY THE LACTATING SHEEP MAMMARY GLAND [1,2]

The contribution of serine to the metabolism of the mammary gland in capacities other than protein synthesis is unknown. Recent reports of Wood *et al.* (6, 7) qualitatively show that serine can be formed in the mammary gland from glucose and glycerol. The conversion most likely occurs by way of phosphoglycerate and phosphohydroxypyruvate (3). A reversal of this reaction sequence could lead to the formation of phosphoglyceraldehyde from serine. Since triose phosphate isomerase is active in the gland (7), it would be possible for serine to contribute to the formation of glycerol.

To study the metabolism of serine by the mammary gland, 90 μc. of serine-3-C^{14} was placed, immediately after milking, into the gland cisterns of the udder of each of two lactating ewes. After 8 hr., the ewes were milked, sacrificed, and the glands removed for analysis. Various substances (Table 1) were isolated from the milk and mammary gland tissue by conventional means and assayed for radioactivity in a thin-window gas-flow G.M. counter. Both milk and mammary tissue were analyzed to eliminate the effect of differences in secretion rates of the various milk components on the distribution of C^{14}.

During the 8-hr. period between the addition of the labeled compound to the udder and the sacrifice of the animal, 94 and 87% of the labeled serine were utilized from the gland cisterns in Experiments A and B, respectively. Of the serine taken up, 16% was recovered from milk and glandular tissue in Experiment A, whereas 36% was recovered from these sources in Experiment B. Of this recovered C^{14}, 98% (Experiment A) and 99% (Experiment B) were located in the proteins of the milk and mammary gland. Table 2 shows the specific activity of the classical protein fractions in the milk from Experiment A.

The specific activity of casein and lactalbumin, in relation to the per cent serine in each (casein 6.3, lactalbumin 4.8), suggests that

TABLE 1

Distribution of recovered C^{14} from serine-3-C^{14} metabolized following administration to the lactating sheep udder[a]

Metabolites isolated	Per cent of metabolized, recovered C^{14}	
Milk	Exp. A	Exp. B
Triglyceride glycerol	Trace	Trace
Triglyceride fatty acids	0.0	0.0
Phospholipids	0.2	0.1
Proteins	33.6	82.9
Lactose	0.0	0.2
Mammary gland		
Triglyceride glycerol	0.4	Trace
Triglyceride fatty acids	0.0	0.0
Phospholipids	0.9	0.1
Proteins	64.7	16.7
Glycogen	Trace	Trace

[a] In Experiment A, 94% of the administered serine was metabolized, of which 16% was recovered in the substances isolated. In Experiment B, 87% of the serine was metabolized, of which 36% was recovered.

TABLE 2

Specific activity of the classical fractions of milk proteins following the arministration of serine-3-C^{14} to the lactating sheep udder (Experiment A)

Protein fraction [a]	Specific activity
	(c.p.m/mg)
Casein	147
Lactoglobulin	49
Lactalbumin	103

[a] Nomenclature as suggested by Reference 4.

[1] Authorized for publication on June 5, 1959, as Paper No. 2368 in the Journal Series of the Pennsylvania Agricultural Experiment Station.
[2] Supported by the U. S. Public Health Service (H3632).

they arose from the same amino acid pool within the mammary gland. The formation of these proteins from the same amino acid pool previously has been suggested by the work of Askonas *et al.* (2). Another report by these workers (1) shows that at least a portion of the immune globulins passes directly from blood to milk. The dilution of synthesized globulin by the inclusion of preformed proteins from the blood could account for the low specific activity of lactoglobulin isolated from the milk in this experiment.

Although the percentage of recovered label in phospholipids is rather small, it indicates the incorporation of serine carbon into this lipid fraction. Since the serine was labeled in carbon 3, ethanolamine or choline formed from serine would still retain the labeled carbon in the molecule. The amount of these nitrogenous compounds present in the phospholipids of milk is very small when contrasted to the much greater quantities of serine in the milk proteins. This large difference in quantities present could explain the distribution of label between these two fractions.

The results indicate that serine did not undergo any major reactions in the mammary gland other than its incorporation into proteins and phospholipids. For serine to have been transformed as previously suggested it would first have to be phosphorylated. This would not appear to be a limiting step in the mammary gland, since the serine incorporated into casein is in the form of the phosphate ester.

Between 64 and 84% of the labeled serine removed from the gland cisterns could not be accounted for in the substances isolated from milk and glandular tissue. It is possible that the major portion of the serine was carried to other tissues of the body by blood traversing the mammary gland. In this regard, it is interesting to note the results of Rogers and Kleiber (5) with labeled acetate introduced in the same manner. These workers found that the majority of labeled acetate was utilized by the quarter into which it was placed. Thus, there may be a great difference in the rates of utilization of serine and acetate by the mammary gland. A slower entrance of serine into the metabolism of the gland could permit more to be carried from the udder without alteration. Another possibility, to account for the loss of C^{14} label, would be the conversion of large quantities of serine to glycine. In this reaction, the labeled carbon would be removed from the serine molecule.

The method of placing labeled compounds directly into the gland cistern, as introduced by Rogers and Kleiber (5), appears to be an effective means of insuring, at least for water-soluble materials, that the desired metabolite is presented primarily to the mammary tissue without alteration by other tissues of the body.

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OUR INDUSTRY TODAY

BRUCELLOSIS ERADICATION MOVES AHEAD AT ACCELERATED PACE

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At the American Dairy Science Association's annual meeting in Indiana in 1940, the Dairy Cattle Health Committee of the Association was born in the Extension Section. Its first committee report was presented the following year at Vermont, before a joint session of the Extension and Production groups. From that time until the present, this committee, later becoming a joint committee of Extension and Production, has made its reports regularly. Brucellosis eradication in the United States from the start has been a prime objective. Now, in 1959, this once costly cattle disease is definitely and surely being brought under control—eradicated.

As of June 30, 1959, 19 states, the Virgin Islands, and Puerto Rico were designated as modified-certified brucellosis-free areas, a status meaning that over 99% of the cattle and over 95% of the herds are brucellosis-free. In addition to these areas, 829 counties in 29 other states also are certified. The most recent states to gain this enviable position were Nevada, Arizona, and Maryland. The first state was North Carolina in 1942, followed by New Hampshire in 1949.

The accompanying map of the United States shows the states and counties (in black) that were on the certified list, March 31. Education, legislation, testing, and calf-vaccination have enabled these areas to reach these goals.

STATE PROGRAMS RECEIVE STUDY

Last summer and fall (1958), I had occasion, while on sabbatical leave from Cornell, to visit 18 western and southern states to study the livestock health problems and programs. Three of these states were certified: Washington, Utah, and New Mexico. Many of the others visited, while not certified, did have counties that had reached this status. Montana for instance had 50 of its 56 counties on the list. In Arizona, all but Maricopa County had reached this goal.

California, with only one county then certified (Del Norte in the north), was steadily pushing southward with its cattle-testing program. It was anticipated that by the end of 1959 about 36 counties would be certified, and that by December 31, 1960, the entire state would attain this coveted recognition. Texas

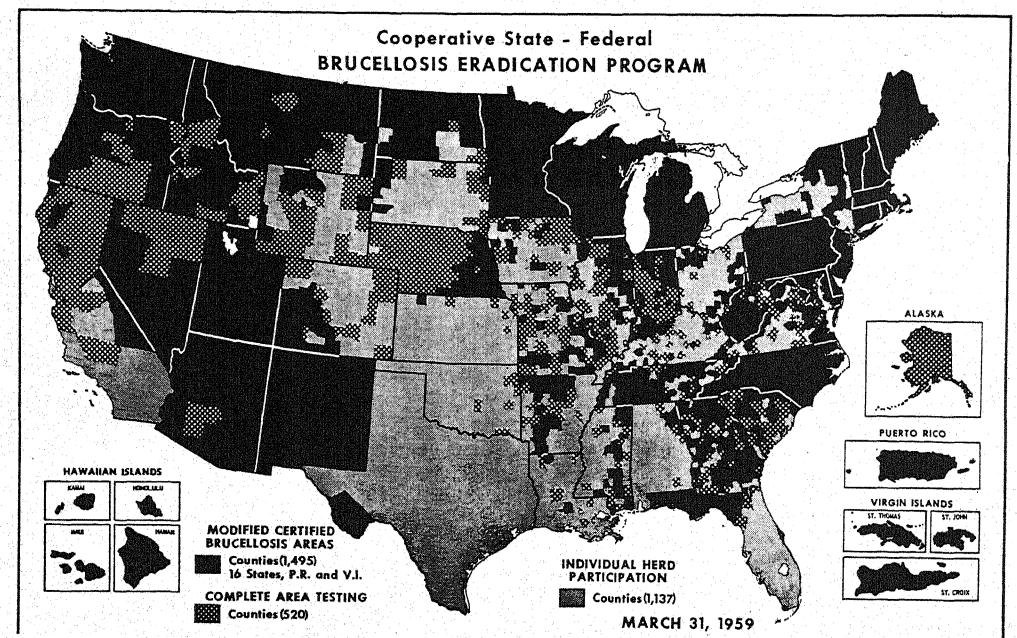


FIG. 1. United States map. Nineteen states have been approved as modified-certified brucellosis-free areas. Arizona, Maryland, and Nevada were recently added to the list.

has been slow to embark upon an official plan for brucellosis eradication. It is now moving forward with the support of the Texas Animal Health Council, and the Extension Service is actively promoting educational programs to stimulate eradication interest. Three counties in the southwestern part of the state are certified. However, since Texas is a big state of 254 counties, 4 yr. may elapse before state certification can be achieved.

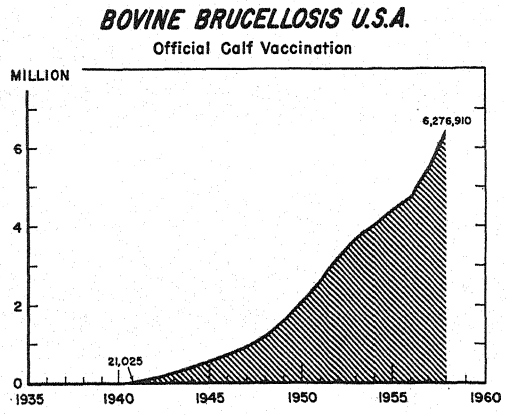


FIG. 2. Calf vaccinations. Calf vaccinations with Strain 19 vaccine have increased steadily since officially approved in 1940.

In all of the states visited upon this extended survey, brucellosis eradication and state certification were major projects and goals. The chief factors limiting more rapid progress appeared to be a lack of funds and insufficient numbers of veterinarians to keep the work progressing as rapidly as cattle owners and officials desire. However, to meet the personnel problems, local practicing veterinarians are being utilized upon a fee basis in many states, to supplement the services of state and federally employed veterinarians. This is speeding certification progress in these areas.

MILK RING TEST, A BLOOD TEST SUPPLEMENT

The blood agglutination test has been the basic test for determining the presence or absence of brucellosis. With the advent and development of the milk ring test (ABR test) in recent years, another tool in the fight against this disease has emerged. Without its availability and utilization in the major dairy states now certified, Minnesota, Wisconsin, and Pennsylvania, it is doubtful if these states could have achieved their objectives at the time they did.

The milk ring test is a rapid, reliable, low-cost method for screening the brucellosis-suspicious herds from the negative herds. In comparing the accuracy of the milk ring test with the blood test, 3,615 herds and 73,155 cattle in two Wisconsin counties were tested. The ring test in this trial missed only one herd

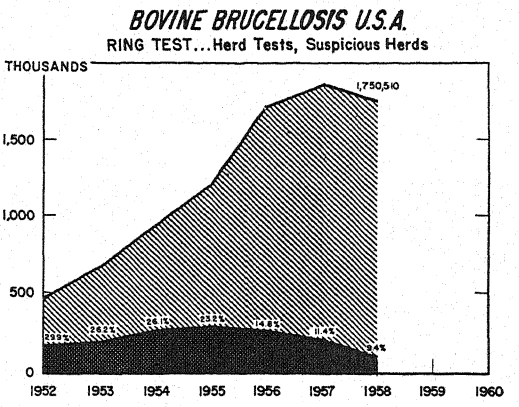


FIG. 3. Ring tests. Milk ring testing as a blood test supplement has made a major contribution to the elimination of brucellosis in dairy sections.

that contained a lone lactating reactor that proved to be a shedder of brucella organisms.

On the basis of cost, to locate each herd containing reactors in Calumet County, the expense was $1,014 by the blood test and $104 by the milk ring test method. In Oconto County the costs were: blood testing $536; ring testing $102.

Most states are using the milk ring test as routine procedure for dairy herds. When infected herds are suspected as a result of the ring test, the blood test is applied to locate the infected individuals. Reactors, if found, are tagged, branded, and removed to slaughter with or without indemnities, in accordance with the state plan of procedure. Thirty states pay some reactor indemnities.

The ring test is not accepted for area certification purposes in all states. This is true in states like Nebraska where many herds are mixed herds, beef and dairy cattle. The ring test used alone for these mixed herds does not give a true herd status. Therefore, under these conditions, only the results from blood tests are being accepted. As a rule, the dairy states utilize both blood tests and ring tests for area certification.

Another milk test known as the milk whey test has been developed in California. This new test is being tried out under field conditions in Marin and Sanoma counties, to determine its accuracy compared to the blood test in locating infected animals. These field trials are being watched with interest by federal and state sanitary officials, and by the research people who developed this test.

SIX MILLION CALVES VACCINATED IN 1958

Calf vaccination with Strain 19 vaccine, after extensive field work in the late 1930's, was given official approval for use by the United States Department of Agriculture in December, 1940. Since that time, calf vaccina-

tion has become a major brucellosis control force in many states. Over 6,000,000 calves, 41% of those eligible, were officially vaccinated in 1958, according to a report of the United States Agricultural Research Service, Animal Disease Eradication Division. Each year, more calves receive these protective shots.

Last year, Wisconsin vaccinated 529,000 calves; Nebraska, 441,000; Montana, 308,000; California, 396,000; Missouri, 306,000; New York, 261,000; Iowa, 261,000; South Dakota, 273,000, and Kansas, 228,000. Since 1941, official records show that 42 million calves in the United States have received Strain 19 vaccine. So convinced are many buyers of replacement cattle of the protective value of calf vaccination that they refuse to consider the purchase of unvaccinated animals. In some areas, dairy nonvaccinates are selling at a discount price of $40 to $50 a head below the price of the vaccinates.

Vaccination has been a great boon to the brucellosis eradication effort in this country, especially in areas previously heavily infected as many intensive dairy areas once were, and in the range states where many ranchers have frowned upon blood testing as a method of brucellosis control. Fortunately, vaccination came along to fill this gap where test-and-slaughter procedures apparently failed to satisfy.

A problem arising from calf vaccination, so well has it met the needs in most range states, is that some people desire to discard the blood test and substitute vaccination as the major program method. Such a shift, in the opinion of brucellosis specialists, is a backward step from the present program aimed at complete eradication. Vaccination may largely control the economic effects of brucellosis, they say, but will not eradicate it. Most folks prefer not to live with this disease, so eventual removal of all reactors still remains state and federal long-time policy.

UNIFORM METHODS AND RULES ADOPTED

For years, efforts have been put forth to formulate a brucellosis eradication program that meets the diverse livestock conditions prevailing in the various states and regions. The National Brucellosis Committee and the United States Livestock Sanitary Association have worked diligently in past years with federal people, to develop uniform methods and rules that meet local conditions and at the same time are sound from the disease-control standpoint. Such a set of Uniform Methods and Rules now has been adopted and published by the United States Agricultural Research Service, Animal Disease Eradication Division, as Bulletin ARS 91-10. Acceptance of these rules by the states has been general, relating to test interpretations, herd and area certification requirements, and animal identification. Rules for the movement of cattle within certified areas and from one certified area in a state to another certified area within the same state have been approved.

In spite of these many areas of agreement on procedures, the requirements for the movement of breeding cattle between states have not been completely settled. While federal regulations have been established, some states have import requirements even more exacting than the federal rules that must be met by sellers of cattle making interstate shipments. This problem of cattle movement is recognized and the hope exists that uniform interstate health regulations approved by all states will be formulated and adopted soon. Many state officials, at present, favor easing some of the restrictions imposed by individual states that are still retarding the free movement of animals across state lines.

RANGE CATTLE TESTED AT SLAUGHTER

Another recent brucellosis program development that is a step forward, in my opinion, is the blood testing of dry and cull cows on the way to slaughter, rather than on the open range, which is often inconvenient and difficult. Under the plan, a range county can be recertified for another 3-yr. period if at least 15% of the breeding cows going to slaughter centers are blood-tested during the 3-yr. period. Slaughter animals will be tagged to identify their place of origin and, when blood-tested at the market, the herds from which all reactors come will be quickly located and tested. Infected herds discovered by this reactor-tracing procedure will be handled in the customary manner, until infection has been eliminated.

It is said that this new beef cattle market testing program is somewhat comparable in convenience to the milk ring test plan for locating infected dairy herds. However, success will depend a good deal upon the cooperation

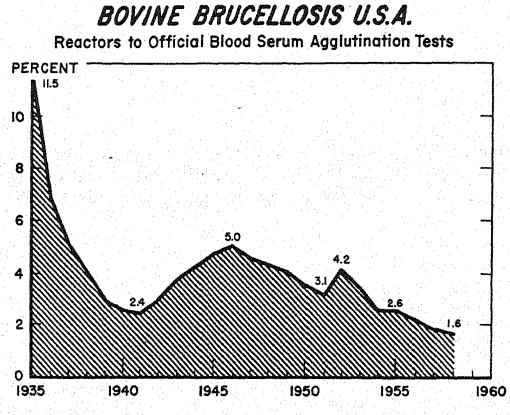


FIG. 4. Reactors. The number of reactors has been steadily declining, but brucellosis is not yet eradicated.

of producers in providing the means for tracing the reactors to the infected herds of origin.

Before adoption this plan was first tried with favorable results in range areas in the State of Washington.

BRUCELLOSIS IN GOATS, SWINE, HUMANS

Often, when thinking of brucellosis eradication, one naturally has cattle in mind as the animal species involved. While the elimination of this disease in cattle is of greatest concern, brucellosis affects other species, including man. It is reported that in 1958, 12,185 goats in 2,320 goat herds received blood agglutination tests. Thirty reactors and 157 suspects were found.

In swine, 2,320 reactors and 3,323 suspects were located among 137,805 hogs in 16,840 herds that were tested. The infection rate was 1.6% reactors and 2.4% suspects. At present, 26 states offer swine brucellosis elimination programs, and the United States Livestock Sanitary Association at its annual meeting last fall suggested a uniform eradication program leading to swine-herd certification for adoption by the states.

To date, swine producers have not become very excited about brucellosis, although some breeders feel it is to their sales advantage to offer stock, especially boars, from certified herds. It appears that as brucellosis is eradicated in cattle, the elimination of the disease in swine will receive increased attention and emphasis. Already, a few states are offering free blood tests to interested swine breeders wishing to test their herds.

As for human brucellosis, the number of cases reported has declined steadily from over 6,000 in 1947 to a little less than 2,000 in 1958. It is believed that the reduced instances of the disease in farm animals during this period have contributed greatly in lessening the infection among humans.

GUARD AGAINST APATHY AS VICTORY NEARS

In conclusion, it can be emphatically said that brucellosis in cattle is being steadily eliminated upon the farms and ranches of the United States. In the year 1958 alone, 493 additional counties were certified, infected herds declined 21%, and the number of disclosed reactors dropped by 10.8% from the previous year. Today, most large milk markets are receiving milk from brucellosis-free herds only, and thousands upon thousands of cattle upon range have received vaccination protection against this costly disease.

As one looks back 25 yr., to 1934, when brucellosis eradication as a national program took root, it can be seen that progress indeed has been remarkable. The chief danger right now, while we are on the threshold of victory, is that a state of apathy and complacency will develop to retard the final eradication effort. This should be vigorously guarded against, as now is no time to relax, feeling that the job has been completed. Continued cooperation from all quarters is demanded, to finish the task of certifying every county and every state. Even then, eternal vigilance will be required for a long time afterwards, in order that reinfection does not rise to mar the gratifying picture of brucellosis-free herds upon every farm. In the future, as in the past, the American Dairy Science Association can lend its helpful support to this worthy public project.

And lest we not forget, healthy cattle still remain the backbone of a profitable dairy industry.

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OUR ASSOCIATION

REPORT TO THE AMERICAN DAIRY SCIENCE ASSOCIATION BY ITS REPRESENTATIVE TO THE NATIONAL RESEARCH COUNCIL 1958-1959

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Annual Meeting—National Research Council. High light of the year's activities was the annual meeting of the National Research Council (NRC) which followed the pattern of the highly successful annual meeting of the previous year. Under this plan all eight divisions of the National Research Council met in three plenary sessions. At the first of these sessions presentations were made on the following topics of broad general interest:

Nature and Conduct of Fellowship Programs in Science, discussed by Warren Weaver.

U. S. Science in Foreign Aid, discussed by George Harrar.

Means and Patterns for the Support of Scientific Research, discussed by Alan Waterman.

The Place of Behavioral Science in the National Research Council, discussed by Clyde Kluckholn and Don Marquis.

Annual Meeting—Division of Biology and Agriculture, Burr Steinbach, Chairman. All 34 of the member scientific societies had representatives present. For the benefit of new representatives, and for the orientation of others, Steinbach outlined the function of the members and the relationship of their societies to the over-all National Academy–National Research Council structure.

The principal function of members of the Division is to examine its work and to try to guide it along the lines of greatest need, interest, or importance to various branches of biology and agriculture, and to science, government, the national welfare, and human welfare in general. Many of the members of the Division also participate in the work of its boards, committees, and panels, through which the experience and wisdom of many American biologists are brought to bear on problems in biology and agriculture.

The Academy–Research Council is enjoined, when called upon, to advise the government, but is completely outside of government control. This is the feature distinguishing it from other advisory agencies, created since World War II (National Science Foundation, President's Science Advisory Committee, etc.). Thus, the NAS-NRC has evolved from a passive body to an aggressive one; it must determine the role it should play in the national and international scene.

The Executive Committee has asserted that the Division must take the lead or participate in broad studies dealing not only with biology or agriculture as natural sciences, or not only with natural sciences extending beyond the boundaries of biology and agriculture into the subjects of other divisions, but with natural and social sciences connected with agriculture and human welfare. The general problem of renewable natural resources is an example, as is that of educational policies conducive to the production of scholars in biology.

W. E. Krauss, Chairman of the Agricultural Board, in keeping with the newer outlook previously expressed, anticipated broadening the scope of the Board's activities to encompass a variety of problems having social and economic aspects, such as:

Population dynamics, land dynamics, and the manpower needed for future production.

Effect of limited or total war on agriculture—decontamination and then restoration of production.

Effect of increasing mechanization and food consumption habits on patterns of production.

Effect of U. S. agriculture on foreign agriculture, and vice versa.

Impact of all factors on the development of a sound agricultural research program.

A grant of $50,000 has been made by the Rockefeller Foundation to support the above program. Applications are encouraged from scientists who may wish to devote two to three years to spearheading and coordinating the work to be done.

Agricultural Research Institute. President Burt Johnson emphasized the unique intermingling among land-grant institution personnel, USDA, and other government agencies, and officials from industry made possible through membership in ARI. Development of forums, advising and helping finance the Agricultural Board, constitute the principal activities of ARI.

Food and Nutrition Board. LeRoy Voris, Executive Secretary, related the establishment of dietary standards, flour enrichment, encouragement of international studies on protein malnutrition, and creation of the Food Protection Committee, as outstanding accomplishments of this Board.

Food Protection Committee. Paul Johnson, Executive Secretary, announced that steps were under way to hold a conference on toxicology, in cooperation with the Committee on Toxicology of the Division of Chemistry and Chemical Technology, for the purpose of promoting toxicology as a scientific discipline separate from pharmacology.

Institute of Laboratory Animal Resources (ILAR). Chairman D. W. Jenkins pointed to the establishment of minimum standards for mice; sponsorship of the Animal Breeders Association for maintenance of standards; establishment of a new committee on cell and tissue culture; and participation in international surveys of laboratory animals, as outstanding accomplishments. The ILAR Washington Office has become an effective information center in this field.

In the general discussion following presentation of the previous reports, the question of usefulness of the Division was raised. Under the new aggressive general policy it was felt that the NRC could continue to have marked influence in guiding major policies affecting scientists. Greater participation, through suggestions by scientific society representatives, and greater recognition by the societies themselves of the potentials of the NRC, would help make this possible.

To this end, a monthly newsletter to all society representatives will be forthcoming. A questionnaire has been sent to each Society, the answers to which will be helpful in bringing the societies and the divisions closer together than does attendance alone by the representative at the annual meeting.

A matter of considerable concern to the Division is the broad problem of use and care of renewable natural resources. It is felt that a standing committee is needed to stimulate basic research in this field and consider from the scientific point of view public policy relating to renewable natural resources. Member society opinion regarding the role of NAS-NRC in this field is solicited.

ACTIVITIES DURING THE YEAR

Members of the Division participated in the procurement of nominees for a U. S. National Committee on the International Union of Biological Sciences. Included in the personnel of this new committee is J. L. Lush, a member of our Society.

Productivity of the Division, as in all scientific endeavors, can be measured in part by publications. These were numerous during the year (see the appendix). Arrangements for procural of the publications listed can be made through Frank L. Campbell, Executive Secretary, Division of Biology and Agriculture, National Academy of Sciences, 2101 Constitution Avenue, Washington 25, D. C.

APPENDIX

Status of Publications Since Annual Meeting, 1958

PUBLISHED	IN PREPARATION
HANDBOOKS ON BIOLOGICAL DATA	
Handbook on Toxicology Vol. III, Insecticides Vol. IV, Tranquilizers Vol. V, Fungicides	Handbook on Circulation
Handbook on Respiration	
BIOLOGY COUNCIL	
The Developmental Biology Conference Series, 1956: Embryonic Nutrition Regeneration in Vertebrates Cytodifferentiation Environmental Influences on Prenatal Development Immunology and Development Physiology of Insect Development Dynamics of Proliferating Tissues Endocrines in Development Mitogenesis Wound Healing and Tissue Repair	
Concepts of Biology (NAS-NRC reprint)	

(APPENDIX — Cont'd)

Published	In Preparation

Educational Policies

Biological Education—A Partial Bibliography

Recommendations on Undergraduate Curricula in the Biological Sciences

Improving College Biology Teaching

Criteria for Preparation and Selection of Science Textbooks

Career Opportunities in Biology (NAS-NRC reprint)

Laboratory and Field Studies in Biology: A Sourcebook for Secondary Schools

Food and Nutrition Board

The Role of Dietary Fat in Human Health

Dietary Fat and Human Health: A Summary Based on the Role of Dietary Fat in Human Health

Recommended Dietary Allowances

Cereal Enrichment in Perspective

Proceedings, Vol. XVIII (Mimeographed)

Food Packaging Materials—Their Composition and Uses

The Safety of Polyoxyethylene (8) Stearate for Use in Food

Insignificant Levels of Chemical Additives in Food Food and Drug Administration, *Cosmetic Law Journal*, 15: 477-479

Evaluation of Protein Nutrition with Emphasis upon Amino Acid Proportionalities

Revision of:
"The Use of Chemical Additives in Food Processing"

Revision of:
"Principles and Procedures for Evaluating the Safety of Intentional Chemical Additives in Foods"

Committee on Photobiology

Research in Photosynthesis
The Gatlinburg Conference, October 25-29, 1955

In press:
Photoperiodism and Related Phenomena in Plants and Animals. The Gatlinburg Conference, October 29–November 2, 1957

Institute of Laboratory Animal Resources

Quarterly News Letter: Information on Laboratory Animals for Research

Animals for Research: A Catalogue of Commercial Sources

A Handbook of Laboratory Animals

Standards for the Transportation of Laboratory Dogs

Agricultural Board and ARI

Research Needs Relating to Agricultural Equipment (Reprint)

Whither Weather, Climate and Agriculture (Reprint)

Nutrient Requirements for Beef Cattle (Revised)

Composition of Cereal Grains and Forages

Races of Maize in Brazil and Other Eastern South American Countries

Proceedings, Seventh Annual Meeting, ARI

U.S.–Canadian Tables of Feed Composition

Methods for the Examination of Poultry Biologics

Methods in Range Research

BOOK REVIEW

INVESTIGATION OF MILK PROTEINS. P. F. D'Achenko. Proceedings of the "Federative Scientific Research Institute of the Dairy Industry (VNIMI)", No. 19, Moscow, 1959. 85 pp., 25 figs., and 38 tables. In Russian. Broche price, 4 Rb. 90 Kop.

This booklet summarizes the results of milk protein research carried out by the members of the above-mentioned institute, together with a review of non-Russian literature on the subject.

The book's title, however, suggests a broader scope than is given by its real content, for 79 pages of the book are devoted to the chemistry of casein; whereas, the remaining six must complete the picture of the whey proteins, of enzymes and low-molecular protein material in milk, and of the protein composition of the fat globule membrane. It is clear that this is by no means a simple task, and that the author failed to accomplish it. As a general survey of our knowledge of the milk proteins, the present review can not sustain a comparison with other treatments of the same subject; for instance, with McMeekin's recent review (Neurath-Bailey, *The Proteins,* Vol. IIA). Rather, it should be considered as a report of the author's own contributions to the chemistry of casein, completed with some results from literature.

Nevertheless, D'Achenko's treatment of casein chemistry is worth while, to be studied by non-Russian, as well as Russian, workers interested in this field.

The book is divided into three chapters: I. Classification of milk proteins; II. Dispersive properties of milk proteins; III. Coagulation of milk proteins.

Chapter I deals with such subjects as heterogeneity, amino acid composition, and titration of casein. The reactions of casein with aldehydes and especially with aldoses are treated extensively.

In Chaper II the state of dispersion of the casein micelles in milk has been studied by the author mainly by light-scattering and electron microscopy. The established distribution of particle dimensions is found to be in fair accordance with the results of other workers.

In Chapter III an extensive comparison is made of the coagulations of the milk proteins by acid, by rennet, and by calcium ions. It is suggested that the precipitation of the milk proteins by calcium ions at 95° C. may find industrial application, because of its completeness.

Finally, the following comments should be made:

Paper electrophoresis of casein can be achieved also without the addition of large quantities of urea. In this connection, the casein composition resulting from the Russian experiments (32.5% α, 67.5% β), therefore, seems highly improbable and certainly does not agree with the results obtained by other workers and by different techniques.

Whether in this instance, as well as in others, the ionic strength of the casein solutions during titration is kept constant remains obscure.

The molecular weights of casein and paracasein, as revealed by light-scattering in 40% urea solution, bear practically no relation to the state of casein, respective of the paracasein in the milk. It is uncertain, therefore, that such measurements could afford some insight into the changes of casein brought about by rennet.

The arguments for a phospho-amidaze action of rennet brought forward by D'Achenko are not very convincing. In fact, they contradict rather than substantiate such an action.

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PREPARATION OF CALCIUM-SENSITIVE α-CASEIN

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SUMMARY

A relatively simple modification of the method for fractionating whole casein in urea solutions is described which gives a calcium-sensitive α-casein. The yield is about 40% of total α-casein. This casein and α-paracasein precipitate to the same extent with $CaCl_2$; 10 m*M* $CaCl_2$ per liter at pH 7 is sufficient to precipitate a 1% solution of each casein. The two caseins can, however, be distinguished by their initial rates of aggregation with lower concentrations of $CaCl_2$ (1.5 to 3.5 m*M* per liter). With low concentrations of $CaCl_2$, whole α-casein aggregates to a greater extent than does calcium-sensitive α-casein. The calcium-sensitive α-casein appears to be homogeneous on electrophoresis at pH 8.5 and 2.3. Very little (less than 1%) of this casein is made soluble by the action of rennin.

The preparation of calcium-sensitive and calcium-insensitive (kappa) casein has been described by Waugh and vonHippel (9). The procedure required an ultracentrifuge. The present investigation was begun when α-caseins, prepared by different individuals by differential solubility in 4.6 *M* urea (4), were found to differ greatly in their sensitivity to calcium ion. In one instance, the product was precipitated almost quantitatively at pH 7.0 with 10 m*M* $CaCl_2$ per liter, similarly to a product prepared by the method of Waugh and vonHippel (9). In other instances, less than 40% of the α-casein was precipitated when the concentration of $CaCl_2$ was as much as 200 m*M* per liter. This paper describes the conditions for preparing either product. When the calcium-sensitive α-casein is prepared, a fraction rich in the calcium-insensitive α-casein is obtained. The method for characterizing the caseins by their quantitative precipitation with $CaCl_2$ is described.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of whole casein. The casein was precipitated from skimmilk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid (3). The precipitated casein was extracted and dried by washing successively with ethanol, acetone, and ether.

Preparation of α-casein. The α-casein, which will be called whole α-casein, was prepared from casein by differential precipitation from urea solutions (4). As a rule, unpurified acid-precipitated casein, stored in frozen condition, served as the starting material. The precipitate was suspended in water, washed several times with water, and dissolved in urea solution. The procedure described by Hipp *et al.* (4) was followed. The final removal of the urea from the casein precipitate by washing with water was facilitated by first breaking up the sticky precipitate suspended in water in a Waring blendor. The final product

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[1] Eastern Utilization Research and Development Division, USDA.

was dried with organic solvents in the same manner as the whole casein. As a rule, the products obtained by this method, although showing some variation, were relatively insensitive to $CaCl_2$ in the precipitation test described later.

Preparation of calcium-sensitive α-casein. Use of the above method for preparing α-casein led in one instance to a product that was very sensitive to $CaCl_2$ (90% precipitated with 10 m*M* $CaCl_2$ per liter). The major variation in the procedure that led to the calcium-sensitive product was the use of decantation instead of centrifugation to separate the α-casein precipitated by diluting the 6.6 *M* urea solution with water to 4.6 *M* urea concentration. This procedure, however, did not always give a calcium-sensitive α-casein (2). Calcium-sensitive products also were occasionally obtained when the precipitate obtained in 3.3 *M* urea was kept at 7° for 18 hr. Similarly, the addition of a small amount of NaOH (6 ml. of 0.1 *N* for 150 g. of wet casein) at this stage in the fractionation sometimes led to a calcium-sensitive product. Calcium-sensitive α-casein was regularly obtained, however, with the following simple modification of the fractionation in urea solutions (4). One hundred and fifty grams (approximately 32 g. dry weight) of wet casein were fractionated at one time. Following the first precipitation (4.6 *M* urea), the concentration of NaCl in the 6.6 *M* urea was doubled from the 1.59 g. used by Hipp *et al.* (4) to 3.18 g. per 150 ml. The α-casein was precipitated (A) with 150 ml. of water in the usual way. The part remaining in solution was recovered to give a calcium-insensitive fraction for future study. The α-casein precipitate (A) was dissolved again in 6.6 *M* urea containing 3.18 g. NaCl per 150 ml. and precipitated again by the addition of water. This product was washed free of urea as described for whole casein, and extracted with the organic solvents. Yield: 7.5 to 8.0 g.

Preparation of α-paracasein. The α-paracasein was prepared by the action of pepsin on α-casein at pH 6.5 in the presence of 15 m*M* $CaCl_2$ per liter. Solution of the calcium-precipitated paracasein was facilitated by the addition of Versene equivalent to the calcium present, and the Ca-versenate was eliminated by reprecipitation at pH 4.7 and dialysis. Other details of the procedure have been described (12).

Precipitation of caseins with calcium chloride. The degree to which casein precipitates with calcium chloride is an important property of casein and it is desirable to characterize each preparation by its quantitative precipitation with a range of $CaCl_2$ concentrations. A test of this type has been described by Nitschmann *et al.* (1, 6). These authors added $CaCl_2$ to a 3% solution of casein. In the present studies, a 1% solution of casein was used and the test has been modified in other minor ways. Each test was performed with several concentrations of $CaCl_2$. These were chosen within the range of 2 to 200 m*M* per liter, depending on the expected calcium-sensitivity of the casein under test. The final solution contained 1% casein, adjusted to pH 7.0 with NaOH and a known concentration of $CaCl_2$ in a test volume of 10 ml. The solution was placed in a bath for 1 hr. at 30° C., then centrifuged for 10 min. at approximately 3,000 × G (International centrifuge, Head No. 233, 15-ml. centrifuge tube, rheostat reading 40). A portion of the supernatant solution was removed and the casein de-

termined by measuring the light absorption at 280 mμ, after clarifying by adding NaOH, or by binding the calcium ion with versene (11). The results obtained with several casein preparations are shown in Figure 1.

Electrophoresis of caseins. Calcium-sensitive caseins had the same mobility as the original α-casein at pH 8.6 on electrophoresis by the Tiselius technique. Electrophoresis at pH 2.3 (5) provides more information about whole α-casein and its fractions. The whole α-casein, prepared by the original urea method of Hipp *et al.* (4), at pH 2.3, shows a major peak with a shoulder on the slow side and also a small amount (about 5%) of a component with a considerably smaller mobility. Electrophoresis of the calcium-sensitive α-casein at pH 2.3 shows only a single symmetrical peak.

Rate of aggregation of the caseins with calcium chloride. Although the calcium-sensitive α-casein, isolated by the present procedure, and paracasein prepared from whole α-casein, both precipitate almost quantitatively with 10 m*M* $CaCl_2$ per liter (see Figure 1), the two are not identical. Experimentally, the two preparations (calcium-sensitive α-casein and α-paracasein) can be dis-

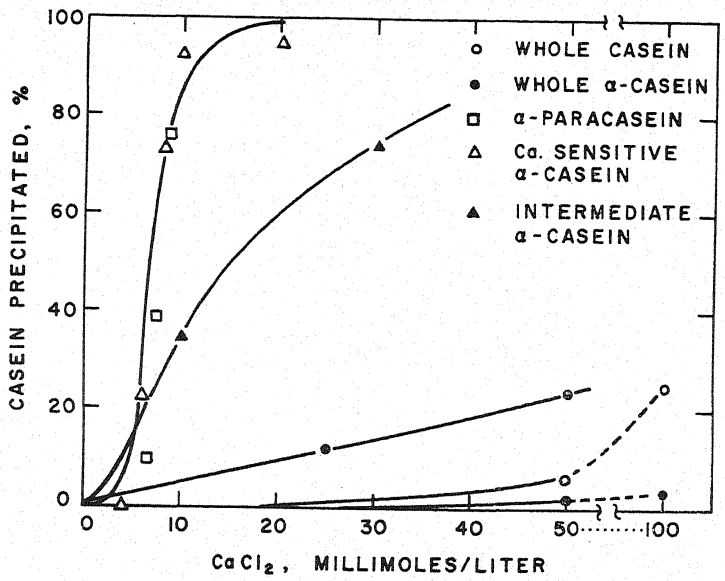


FIG. 1. Precipitation of various casein preparations with calcium chloride at pH 7.0. Results shown for two whole α-casein preparations show the variation observed with the regular procedure.

tinguished by their sensitivity to low concentrations of calcium ions (1.5 to 3.5 m*M* per liter), and the initial course of their aggregation in the presence of calcium ions. Previous spectrophotometric studies (12) have shown that whole α-casein in the presence of $CaCl_2$ will aggregate in a linear manner for more than 30 min. Paracasein, on the other hand, aggregates very rapidly in the first few minutes, then much more slowly, giving a rectilinear type of curve (12).

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α-Paracasein, prepared either from whole α-casein or calcium-sensitive α-casein, gives a similar type of aggregation curve, although less exaggerated, as shown in Figure 2. Also shown in the same figure, calcium-sensitive α-casein gives a linear aggregation curve, similar to whole α-casein.

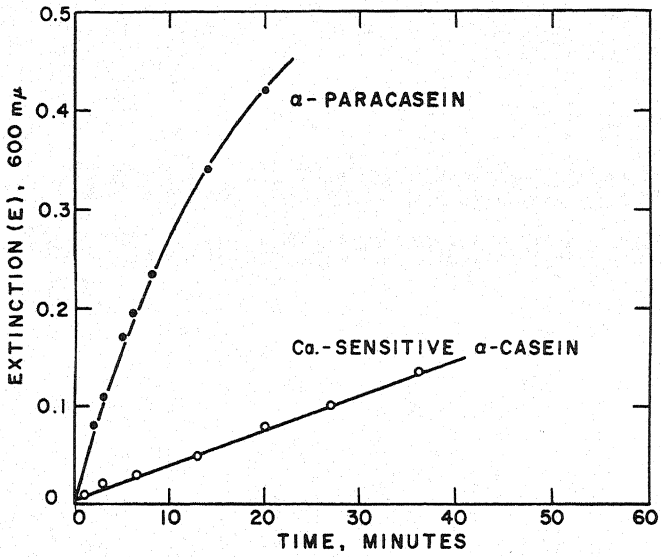


FIG. 2. Rate of aggregation of α-paracasein and calcium-sensitive α-casein in the presence of calcium ions, measured by the light extinction (E) at 600 mμ. The reaction was performed in cacodylate buffer, pH 6.3 at 30° (12). ● : α-paracasein with 1.5 m*M* $CaCl_2$ per liter; ○: Calcium-sensitive α-casein with 3.3 m*M* $CaCl_2$ per liter.

α-Paracasein in the above type of rate experiment is much more sensitive to calcium ion concentration than is the calcium-sensitive α-casein. The respective concentrations used for the experiment illustrated in Figure 2 are 1.5 and 3.3 m*M* $CaCl_2$ per liter. If these preparations and whole α-casein are all compared at pH 6.3 with the same $CaCl_2$ concentration of 3.3 m*M* per liter, an extinction of 0.5 is attained by α-paracasein in 0.5 min., whole α-casein in 10 min., and calcium-sensitive α-casein in more than 2 hr. Because of the difference between whole α-casein and calcium-sensitive α-casein, just the reverse of that expected from precipitation experiments, aggregation experiments were done with both caseins with 3 to 6 m*M* $CaCl_2$ per liter at pH 7.2. Since the aggregate obtained with whole α-casein is stable, the ultracentrifuge was used (45 min. at 105,000 × G) to sediment the aggregates. Neither casein aggregated at this pH (7.2) and temperature (25° C.) with 3 m*M* $CaCl_2$ per liter (at a higher temperature only the whole α-casein became opalescent); with 4 m*M* only 10% of sediment was obtained with the calcium-sensitive α-casein, whereas 22% of sediment was obtained with the whole α-casein. The sedimentation curves crossed between 5.5 and 6.0 m*M*, and at 6 m*M* the respective amounts of sediment were 71 and 63% of the total casein.

The heat-induced increase in the calcium ion aggregation of calcium-sensitive α-casein at lower temperature shows the same type of reversal of aggregation as whole α-casein (10); that is, the higher the initial temperature, the greater the decrease in aggregation on going to a lower temperature. Paracaseins in a similar test reverse very little.

Other properties of calcium-sensitive α-casein. The preparations of calcium-sensitive α-casein obtained by the present procedure regularly contained more phosphorus than whole α-casein. The analysis of a calcium-sensitive preparation was as follows (all on moisture-free basis): P, 1.08%; N, 15.16%; ash, 4.31%.

The various casein preparations were also characterized by the amount that remained soluble when the solution was adjusted to pH 4.7 after rennin had acted on the casein at pH 6.4. The procedure employed and the results have been described (2). The net increase in the soluble portion of whole α-casein was 2.8 to 3.5%, whereas for the calcium-sensitive α-casein, it was only 0.64 to 0.80%.

DISCUSSION

The method just described gives a calcium-sensitive α-casein in relatively good yield, by a simple modification of the frequently used fractionation of whole casein in urea solutions. Further, the effectiveness of the additional NaCl in separating the calcium-sensitive and calcium-insensitive fractions of α-casein suggests that the interaction between these components, at least in part, is due to interaction of electrical charge on the proteins. Increasing the concentration of the salt ion in the environment will reduce such an interaction.

The calcium chloride precipitation test described herein provides a useful characterization for each preparation of whole casein and α-casein. In view of the contribution of the stabilizing fraction to the stability in the presence of calcium ions, it is not surprising that there is some variation in precipitation with $CaCl_2$ from one preparation of casein to another. The $CaCl_2$ test can provide an important characterization for each preparation until the time when a method is available for determining the calcium-stabilizing α-casein content of each sample of milk or preparation of casein. This is even more true of the whole α-casein preparations, since the present study shows that fractionation in urea solutions is sensitive to minor variations in the procedure. If the procedure is standardized, the product will be of constant properties and presumably constant composition, but it is recommended that the $CaCl_2$ test be performed for confirmation.

The phosphorus content for calcium-sensitive casein obtained in the present study is about the same as the 1.1% reported by Waugh (8) for α_s-casein, a calcium-sensitive casein. In the present study, casein made by the procedure of Waugh and vonHippel (9) precipitated with $CaCl_2$ in the same way as the calcium-sensitive casein illustrated in Figure 1. McMeekin *et al.* (5) have shown that a calcium-sensitive casein with about the same phosphorus content and apparently homogeneous on electrophoresis at pH 2.35 could be fractionated in ammonium sulfate solutions to give a product containing 0.85% phosphorus (α_1-casein) and a fraction correspondingly richer in phosphorus.

The amount of casein becoming soluble when it is acted on by rennin can serve to guide the separation of calcium-sensitive and calcium-insensitive products. The former gives very little material soluble with rennin, whereas the latter containing the stabilizing fraction gives a considerable amount. Wake (7) reported that none of a purified preparation of calcium-sensitive casein became soluble when acted on by rennin. The method for preparing this casein was not described. The small amount becoming soluble in the present study may be due to contamination with the stabilizing fraction. This seems unlikely, since the casein was reprecipitated in urea under conditions for separating the stabilizing fraction and the product still gave about the same amount soluble with rennin. An alternative explanation may be that in the rennin test some nonspecific proteolysis may have occurred, not related to the role of the stabilizing fraction in clotting.

The greater amount of aggregate obtained with whole α-casein at low calcium chloride concentrations (about 4 mM per liter at pH 7.2) than with calcium-sensitive α-casein is an interesting phenomenon. When aggregates are obtained with the latter, they coagulate and settle, whereas the former are stable colloids. The explanation for the differences noted is not apparent, but the calcium-sensitive α-casein may bind more calcium ion per unit weight than the whole α-casein and only when saturated with calcium ion is the unstable aggregate formed.

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SOME ESTERASES OF COWS' MILK[1]

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SUMMARY

The influence of different concentrations of the organophosphorus enzyme inhibitors, N,N′-diisopropyl phosphorodiamidofluoridate, diethyl *p*-nitrophenyl thiophosphate, diisopropyl fluorophosphate, and tetraethyl pyrophosphate on the activity of milk enzymes toward phenyl acetate, phenyl propionate, tributyrin, benzyl butyrate, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na was studied, using Warburg manometric techniques at pH 8.0 and 37° C. Raw mixed milk served as the enzyme source.

Three distinct esterases or esterase systems were evident. One was responsible for about 95% of the activity of the milk enzymes toward tributyrin, benzyl butyrate, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na, the second for 60 to 70% of their activity toward phenyl propionate, and the third for about 80% of their activity toward phenyl acetate. Each of these esterases appeared to hydrolyze one or more of the other substrates. A probable classification of these enzymes is outlined.

Evidence which has been accumulating for several years indicates that the hydrolysis of milk fat, as it naturally occurs in milk, results from the activity of more than one enzyme (1, 10, 12-15, 24, 26, 27). To date, however, no clear-cut means of differentiating such esterases have been presented. Consequently, no method of assay for individual esterases has been available. Montoure *et al.* (18) did report the presence of a cholinesterase in colostrum, but did not establish that this enzyme was commonly present in normal milk. This cholinesterase was shown to hydrolyze tributyrin at an appreciable rate.

Substantial progress has been made in the differentiation of lipases, esterases, and cholinesterases of pancreas, blood, and other body tissues and fluids of a number of animal species (2-6, 16, 17, 19-22). This progress has been made possible, largely, by the use of a number of organophosphorus enzyme inhibitors and a variety of pure esters. The procedure most commonly followed has been to compare the enzyme action of the tissue or fluid under study on different esters, after incubation with definite concentrations of a specific organophosphorus inhibitor for a specified time. Thus, it has been possible to recognize individual esterases on the basis of their sensitivity to, or tolerance of, different inhibitors and their specificity toward different esters.

The authors believed that these procedures might be useful also in differentiating the lipases and esterases of milk involved in milk fat hydrolysis. They believed that if some basis could be found for identifying or differentiating even one or two of the enzymes involved, methods could then be worked out to study other properties of such enzymes. A knowledge of the major properties of an individual esterase might then make it possible to determine its role in milk fat hydrolysis and rancid flavor development. To this end the work described below was undertaken.

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While this work was in progress, Augustinsson (7), in a preliminary report, showed that the plasma of vertebrates, in general, contains three types of esterases: A-esterases (arylesterases), B-esterases (aliphatic esterases, ali-esterases, or lipases), and C-esterases (cholinesterases). Later, Augustinsson and Olsson (8, 9), in a more detailed report on the esterases of swine plasma and milk, showed that the C-esterase was the only one of these enzymes present in important amounts in swine milk, though traces of the other two also were present.

EXPERIMENTAL METHODS

Collection and care of milk samples. Samples were obtained from individual tanks (100-gal.) of well-mixed raw milk from the College herd. They were brought to the laboratory within a few minutes after collection, divided into the needed number of portions, gassed with nitrogen for 20 min. (11) while standing in a bath of ice water, sealed in an atmosphere of nitrogen, and stored for no longer than 6 to 8 hr. in a refrigerator (0–5° C.) until needed. Amber-glass Pyrex bottles were used for collection and storage of samples. Vat samples were chosen for this work, so that individual cow variations would be largely eliminated and samples would represent milk from quite a number of cows. It was realized, however, that some loss in esterase activity might have occurred prior to collection and gassing.

Substrates. Substrates used in this work were phenyl acetate, phenyl propionate, and phenyl butyrate, the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na (all synthesized in this laboratory), benzyl butyrate (Givaudan-Delawanna, Inc.), and tri-*n*-butyrin (Eastman). Final concentration of these substrates in the reaction mixtures was 0.006 *M* for the two esters of 2-naphthol-6-SO_3Na and 0.1 *M* for the others. During the early part of this work, substrates were prepared in the form of 1.0 *M* emulsions in 16% (w/v) Triton X-100 (Rohm & Haas), with the exception of the esters of 2-naphthol-6-SO_3Na. The latter were prepared as 0.06 *M* solutions in the same concentration of Triton X-100. Later, it was found possible to prepare more stable emulsions in 3% (w/v) Triton X-155. This material was used in preparing substrate emulsions for most of the work reported below.

Inhibitors. Inhibitors used were diethyl *p*-nitrophenyl thiophosphate (Parathion, Monsanto), N,N′-diisopropyl phosphorodiamidofluoridate (Isopestox, Fisons Pest Control, Ltd.), diisopropyl fluorophosphate (DFP, Ealing Corp.), and tetraethyl pyrophosphate (TEPP, Bios Laboratories). Some of these inhibitors (Parathion and DFP) were not soluble at the higher concentrations required; hence, all were prepared as solutions or emulsions in 1% (w/v) Triton X-100. Inhibitors were in contact with the milk, at the desired concentration, for 30 to 50 min. before tipping in the substrate.

Measurement of esterase activity. Esterase activity was measured manometrically, using Warburg techniques, in a manner similar to that described by Nachmansohn and Rothenberg (23). The main compartment of the Warburg flask contained 1.0 ml. milk, 1.5 ml. $NaHCO_3$ buffer, and 1.0 ml. of either water, 1% (w/v) Triton X-100, or inhibitor solution (whichever was dictated by the

experiment); 0.4 ml. of substrate emulsion and 0.1 ml. $NaHCO_3$ were placed in the side arm. Flasks were gassed 10 min. with a mixture of 5% CO_2 and 95% N_2 and equilibrated for at least 10 min. The contents of the side arm were tipped into the main compartment at zero-time. Thus, the total fluid volume was 4.0 ml. Final pH was 8.0, calculated from the Henderson-Hasselback equation (28), except in those trials where the influence of pH was being investigated. Incubation was for 30 or 60 min., with readings at 10- or 20-min. intervals, respectively. Suitable blanks, containing heated ($75 \pm 5°$ C. for 10 min.) milk, were provided at all times to correct for nonenzymatic hydrolysis of the substrates and a slight tipping error. A number of trials with different samples of unheated milk, without added substrate or inhibitor, resulted in negligible blank values; therefore, hydrolysis of the milk fat was disregarded. All observations were made in duplicate.

Randomization. The order in which the various treatments (inhibitor concentrations) were assigned to flasks for each individual run of the Warburg was randomized, as was also the order in which the substrates were tested on any given day. In those experiments where two or more inhibitors were under comparative study, the order in which they were used also was randomized.

Calculation of results. Duplicate blank values were averaged and these averages subtracted from the appropriate individual duplicate values, to arrive at duplicate net activity values. These latter values for the controls (containing no inhibitor) were averaged and from the averages so obtained were subtracted the net activity values for the various levels of inhibitor. This procedure gave duplicate values for net inhibited activity for each inhibitor concentration–substrate combination. These values were then expressed as percentage of the appropriate controls. In this manner, duplicate per cent inhibition values were arrived at for each combination of substrate, inhibitor, inhibitor concentration, and milk sample.

RESULTS

Preliminary experiments with Parathion showed substantially greater inhibition of esterase activity when phenyl butyrate served as substrate than when benzyl butyrate, tributyrin, or the butyryl or stearoyls esters of 2-naphthol-6-SO_3Na were used. Parathion at concentrations of 10^{-5}, 10^{-4}, and 10^{-3} *M* inhibited the activity toward phenyl butyrate 55, 81, and 95%, respectively. These same concentrations of Parathion produced no more than 4, 19, and 81% inhibition, respectively, with any of the other substrates.

Isopoestox, at concentrations ranging from 10^{-7} to 10^{-3} *M*, was then tested, using the substrates mentioned in the preceding paragraph. This compound inhibited 75% of the phenyl butyrate activity at 10^{-4} *M* concentration; whereas, no more than 5% of the activity toward any of the other substrates was inhibited. A somewhat disconcerting observation from this work was that at 10^{-3} *M* concentration of Isopestox, inhibition of the activity toward phenyl butyrate dropped to 44%. This phenomenon was studied in more detail in later work. It was found that 10^{-2} *M* Isopestox resulted in even less inhibition toward phenyl butyrate. When benzyl butyrate, tributyrin, and the stearoyl ester of 2-naphthol-6-SO_3Na

were tested with 10^{-2} *M* Isopestox, activation rather than inhibition resulted. In the case of the butyryl ester of 2-naphthol-6-SO_3Na, which was in complete solution, 10^{-2} *M* Isopestox produced about 3% inhibition.

A brief study was made of the influence of pH on the activity of the enzyme-hydrolyzing phenyl butyrate and inhibited by 10^{-5} *M* Parathion. The activity increased steadily from pH 6.0 to 8.0. The maximum upper limit of usefulness of the Warburg Method is pH 8.0.

The activity of this Parathion-sensitive (10^{-5} *M*) enzyme toward phenyl butyrate, phenyl propionate, and phenyl acetate was then compared at pH 8.0. It was found that the enzyme hydrolyzed the propionate about twice as fast as the butyrate, whereas it hydrolyzed the acetate more slowly. During the course of this experiment, however, total hydrolysis of phenyl acetate in the controls was five times that of the phenyl butyrate and more than twice that of the phenyl propionate.

During preliminary trials, in which DFP and TEPP were tested at concentrations from 10^{-7} to 10^{-3} *M* with phenyl acetate, phenyl propionate, benzyl butyrate, tributyrin, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na serving as substrates, it became apparent that phenyl acetate showed a different inhibition pattern from any of the others. Even the highest inhibitor concconcentration (10^{-3} *M*) produced, on the average, less than 10% inhibition of the activity toward phenyl acetate. This same inhibitor concentration was sufficient to inhibit 80 to 90% of the activity toward benzyl butyrate, tributyrin, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na, and about 95% of the activity toward phenyl propionate. On the other hand, 10^{-6} *M* concentrations of either DFP or TEPP inhibited 60 to 70% of the activity toward phenyl propionate, while showing less than 10% inhibition toward all of the other substrates.

This preliminary work led to a more comprehensive experiment, extending over several months. In this experiment, the inhibitory action of Isopestox, Parathion, DFP, and TEPP at concentrations from 10^{-7} to 10^{-2} *M* were compared, using only the three substrates, tributyrin, phenyl propionate, and phenyl acetate. The work involved precluded the testing of all inhibitors at all concentrations on the three substrates, using a single sample of milk. Consequently, sample variation may have introduced some error.

Table 1 presents a summary of the work of this experiment. The average values for per cent inhibition reported in this table are directly comparable horizontally, since the same samples of milk and the same inhibitor preparations were used. Sample variation, mentioned in the previous paragraph, may be responsible for a small part of the differences evident upon vertical comparison. A total of 84 different samples was involved in this experiment. The standard error for each average per cent inhibition value shown in the table was calculated ($N = 2 \times$ No. of samples) and is also presented, so that the significance of the per cent inhibition values may be readily assessed. Figures 1, 2, and 3 present graphically the per cent inhibition values shown in Table 1. The very marked differences between inhibitors, inhibitor concentrations, and substrates are more readily visualized by reference to these figures.

TABLE 1

Average percentage inhibition of activity of milk toward tributryrin, phenyl propionate, and phenyl acetate after exposure to 10^{-7} to 10^{-2} M concentrations of Isopestox, Parathion, DFP, or TEPP for 30 minutes

Inhibitor	pI[a]	No. of samples	Substrate: Tributyrin %	Tributyrin S.E.	Phenyl propionate %	Phenyl propionate S.E.	Phenyl acetate %	Phenyl acetate S.E.
Isopestox	7	8	0.4	0.8	4.0	2.2	−0.7	1.6
	6	8	1.4	0.8	8.7	2.6	0.7	1.9
	5	8	0.9	1.2	23.8	2.4	4.9	1.7
	4	8	2.7	1.3	52.8	3.4	10.7	1.5
	3	13	1.0	0.8	52.8	2.3	2.6	1.8
	2	5	1.7	3.1	21.1	2.3	−13.7	2.8
Parathion	7	8	0.7	1.2	2.8	4.5	−0.7	1.6
	6	8	0.8	0.9	13.5	3.3	1.4	1.1
	5	8	2.8	0.9	39.0	3.0	5.1	1.9
	4	8	13.1	1.1	67.8	2.5	8.8	1.3
	3	13	66.4	1.1	80.9	2.2	14.8	1.6
	2	5	99.3	0.9	82.5	4.3	21.3	3.5
DFP	7	8	2.3	0.6	57.7	2.8	7.3	1.6
	6	8	2.2	0.7	70.6	2.5	7.4	1.9
	5	8	3.3	0.7	73.0	2.3	9.7	1.4
	4	8	16.0	1.1	75.6	3.5	7.6	1.4
	3	13	73.5	1.8	78.5	2.0	10.6	0.9
	2	5	98.4	0.8	67.1	5.0	22.5	3.3
TEPP	7	8	1.6	1.0	62.1	2.3	6.4	1.6
	6	8	2.0	0.9	72.6	2.8	11.5	1.7
	5	8	4.4	0.7	70.8	2.2	10.8	0.9
	4	8	22.1	1.9	73.8	2.5	14.5	1.6
	3	13	84.9	2.0	71.8	2.7	6.0	1.2
	2	5	90.3	0.9	40.5	6.4	−0.4	2.6

[a]pI = negative logarithm of molar inhibitor concentration.

DISCUSSION

The preliminary experiments with Parathion and Isopestox established that one of the esterases in milk was responsible for a large part (70%) of the activity toward phenyl butyrate. Furthermore, the results indicated that this enzyme was relatively inactive toward benzyl butyrate, tributyrin, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na. It was also established in the preliminary work that this esterase hydrolyzed phenyl propionate more rapidly than phenyl butyrate or phenyl acetate.

Following completion of the preliminary work with DFP and TEPP it was apparent that milk contained at least three esterases. One was responsible for most of the hydrolysis of tributyrin, benzyl butyrate, and the butyryl and stearoyl esters of 2-naphthol-6-SO_3Na, and was inhibited by higher concentrations of Parathion, DFP, and TEPP, but not by Isopestox. The second was active toward phenyl propionate and was readily inhibited by the organophosphorus compounds. The third esterase was largely responsible for the hydrolysis of phenyl acetate and was resistant to all the inhibitors tested.

The existence of these three esterases is clearly evident from an examination of the curves shown in Figures 1, 2, and 3. Figure 1, for instance, shows that

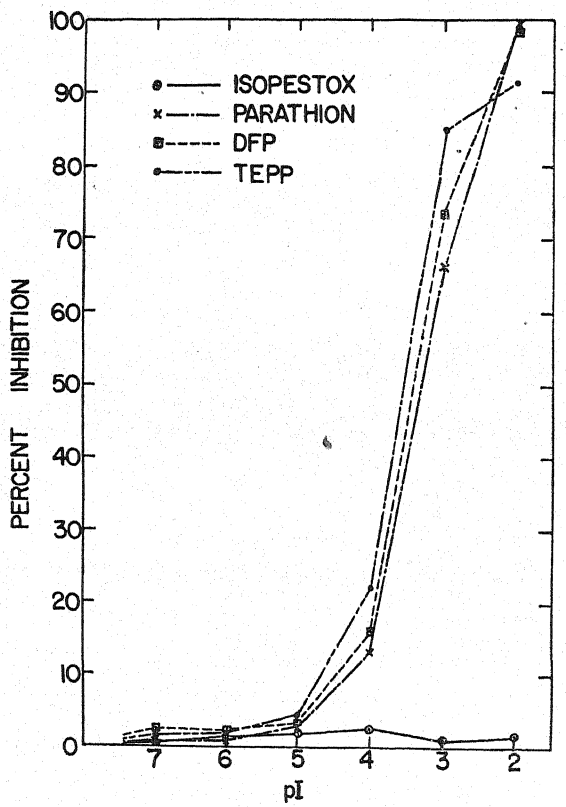
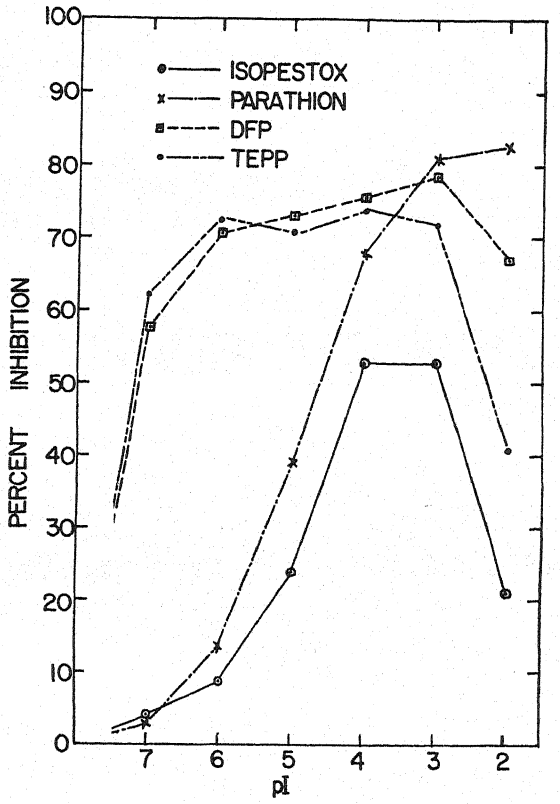


FIG. 1. Influence of inhibitor and inhibitor concentration on inhibition of activity of milk toward tributyrin. pI = negative logarithm of molar inhibitor concentration.

Isopestox, even in relatively high concentration (10^{-2} *M*), inhibited only 2–3% of the activity toward tributyrin. This same concentration of the three other inhibitors resulted in almost complete inhibition. However, these three inhibitors (Parathion, Isopestox, and DFP) did not inhibit appreciably until concentrations of 10^{-4} *M* or higher were used. Below the latter concentration they inhibited to about the same extent as did Isopestox. When phenyl propionate served as the substrate, a concentration of 10^{-4} *M* Isopestox was sufficient to cause over 50% inhibition (Figure 2). Low concentrations (10^{-7} to 10^{-6} *M*) of DFP and TEPP resulted in 60 to 70% inhibition toward phenyl propionate, but even the highest concentration of Parathion (10^{-2} *M*) only slightly exceeded 80% inhibition. The remaining activity (about 20%) toward phenyl propionate was due, no doubt, to the enzyme responsible for about 80% of the activity of milk toward phenyl acetate (Figure 3).

The peculiar downward trend in the curves for Isopestox and TEPP at the higher concentrations in Figures 2 and 3 must have some logical explanation. Limited evidence suggests that these compounds, in relatively high concentrations, appreciably increase the solubility of the substrates. This results in a higher



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FIG. 2. Influence of inhibitor and inhibitor concentration on inhibition of activity of milk toward phenyl propionate. pI = negative logarithm of molar inhibitor concentration.

effective substrate concentration and greater activity on the part of the phenyl acetate enzyme, which is not inhibited by these compounds.

Richter and Croft (25), Aldridge (3, 4, 5) and, more recently, Augustinsson (7) and Augustinsson and Olsson (8, 9) have classified the esterases of blood plasma. The latter investigators have applied this classification to sows' milk. Using the system suggested by these authors, the esterases identified in this work may be classified as follows:

(1) A-esterase (arylesterase): The enzyme of milk responsible for the major portion (70–80%) of the activity toward phenyl acetate (Fiure 3) unquestionably belongs to this class. It showed practically no activity toward tributyrin but did hydrolyze phenyl propionate slowly. It was remarkably resistant to the organophosphorus inhibitors used in this study.

(2) B-esterase (aliphatic esterase, ali-esterase, lipase): The esterase hydrolyzing tributyrin (Figure 1) appears to belong in this class. Examination of Figures 2 and 3 suggests that this enzyme does hydrolyze phenyl propionate and acetate slowly. The preliminary experiments, described earlier in this report, indicate that most of the activity of milk toward benzyl butyrate and the butyryl

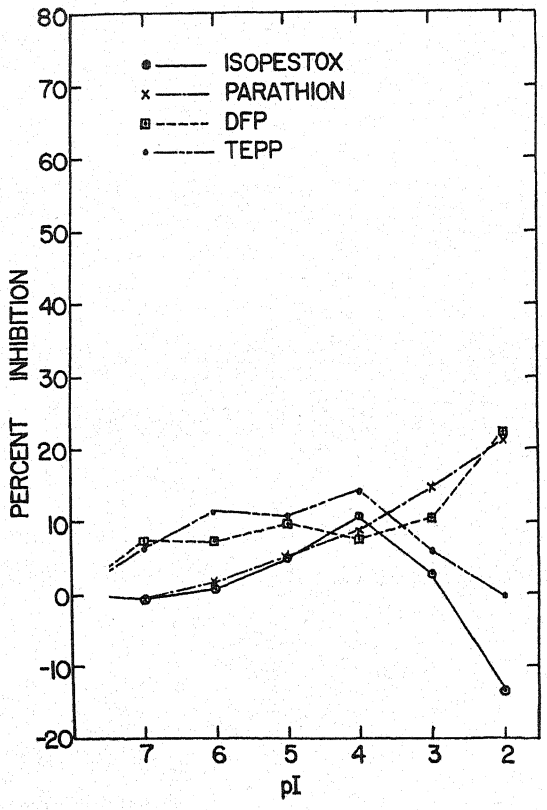


FIG. 3. Influence of inhibitor and inhibitor concentration on inhibition of activity of milk toward phenyl acetate. pI = negative logarithm of molar inhibitor concentration.

and stearoyl esters of 2-naphthol-6-SO_3Na could be attributed to this enzyme. Other data, not presented here, show that hydrolysis of the methyl esters of *n*-fatty acids by milk also is due to this enzyme. It will tolerate up to 10^{-5} *M* Parathion, DFP, and TEPP, but higher concentrations inhibit. Isopestox, up to a concentration of 10^{-2} *M*, is without effect on this enzyme.

(3) C-esterase (cholinesterase): The enzyme of milk responsible for about 70% of its activity toward phenyl propionate (Figure 2), 10% of its activity toward phenyl acetate (Figure 3), and 3% of its activity toward tributyrin (Figure 1) may belong to this class. Additional work will be required to determine whether this is a cholinesterase and whether it is the same enzyme as that found in cows' colostrum by Montoure *et al.* (18).

Each of the above-mentioned enzymes (or groups of enzymes) can be assayed for independently, by varying the inhibitor concentration and substrate. The A-esterase can be estimated by using phenyl acetate as substrate in the presence of 10^{-2} *M* Parathion. On the other hand, the B-esterase will be measured if tributyrin serves as the substrate and the assay is carried out in the presence of 10^{-6} *M* DFP or TEPP. Finally, the C-esterase may be assayed for by using

phenyl propionate as substrate. This assay, however, is slightly more complicated, because all three enzymes hydrolyze this substrate to some extent. In this instance, one must determine the activity in the absence of inhibitor and, simultaneously, in the presence of 10^{-6} *M* DFP or TEPP. The difference between these two values, i.e., the inhibited activity, respresents the activity contributed by the C-esterase.

One might speculate at length on the role of these enzymes in the development of rancid flavor in dairy products and in the synthesis of milk within the udder. It would be more appropriate, however, to withhold such speculation until more facts are available. In this connection, two distinctly different avenues of research present themselves. The first is a study of the factors influencing the concentration of each of these esterases in milk and the relationship of spontaneous rancidity to the concentration of each. A second approach is concentration and purification of each esterase, followed by a detailed study of the physical and chemical properties of each and of the reactions catalyzed by each. Both of these lines of research are presently under way in this laboratory.

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TOTAL MONOGLYCERIDE CONTENT OF SOME DAIRY PRODUCTS

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SUMMARY

The average total monoglyceride contents of several dairy products reported as m*M*/100 g. of fat were: fresh, raw milk—0.077; pasteurized whole milk—0.145; homogenized milk—0.206; 40% cream—0.119; butter—0.189; and Blue cheese—0.354. Some of the implications of the monoglyceride content of dairy products are discussed.

Monoglycerides have been shown to lower the surface tension of milk (2) and to depress the foaming of skimmilk (1). They are added to ice cream mix as emulsifiers. Monoglycerides are known to be present in lipolyzed milk (5, 6), but as yet no report has appeared on the content of these surface-active compounds in normal milk and other dairy products. This information is reported in the present paper.

EXPERIMENTAL PROCEDURE

In general, the estimation and extraction procedures for milk and cream were those previously described for lipolyzed milk (6), except that the quantities of silicic acid and chloroform were increased proportionately in relation to the size of the sample. Some further modifications were necessary for the other dairy products. It was found that when relatively fresh products were analyzed, a 2-g. sample of fat was required. When products other than milk or cream were extracted, the silica-gel was prepared in such a way as to approximate the extraction conditions for milk. In effect, the products were reconstituted so that the sample on the column had the fat-water relationship of whole milk.

Milk. Fifty milliliters were extracted as previously described and the solvent was removed. The fat was rinsed with chloroform into a tared 100-ml. evaporating flask, the solvent removed by a flash evaporator, the flask placed in a vacuum desiccator overnight, and then reweighed. The fat was quantitatively transferred to a 10-ml. volumetric flask with chloroform and the monoglycerides estimated as previously described (5). It was noted that if more than 2.5 g. of fat were placed in the flask, some of the fat would separate and rise to the top during the subsequent analysis. Successful estimation of monoglycerides depends upon the maintenance of a single phase during the test.

Butter. About 2.0 g. were weighed on a watch glass and then scraped and rinsed with 90% chloroform–10% ethanol eluting solvent into a casserole containing prepared silica-gel. The silica-gel was prepared by adding enough silicic acid to 45 ml. of water to make a free running powder. After extraction the fat was treated as described for milk.

Blue cheese. About 1.0 g. was weighed on a watch glass, scraped and rinsed into a casserole containing about 10 g. of dry silicic acid, and ground. Silica-

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gel, prepared as for butter, was mixed with the ground cheese, and extraction and estimation proceeded as described above.

Cream. A volume of sample was extracted which contained about 2.0 g. of fat. Recoveries of monoglycerides from butter, Blue cheese, or cream were not determined. Since these samples were reconstituted to resemble milk, the recovery of monoglycerides from these products should be quantitative (4).

The fresh, uncooled milk samples were extracted within 1 hr. after they were obtained from individual animals in the Department herd. The butter samples were obtained from retail outlets and were scored by two judges. The pasteurized and homogenized milk and some of the cream samples were obtained from the Department plant. The rest of the cream samples were taken from a bulk commercial source and were of unknown age. The Blue cheese samples were purchased from a retail outlet.

The fat from several fresh raw milk samples was saponified, the glycerol removed by silicic acid column treatment (6), and the resultant fatty acids and nonsaponifiable material analyzed for monoglycerides. This was done to confirm the presence of monoglycerides in the fresh raw samples, since saponification would completely remove monoglycerides detectable by the method used (6).

RESULTS AND DISCUSSION

The monoglyceride contents of the various products analyzed are presented in Table 1. The data show considerable differences in content which perhaps can be largely explained by the origin and history of the samples. The fresh raw samples were obtained from the University herd and were extracted as rapidly as possible. Since the extraction treatment inactivates lipase, it is believed that the monoglycerides found represent approximately the amount in

TABLE 1

Total monoglyceride content of various dairy products

Fresh raw milk	Pasteurized whole milk	Homogenized milk	40% Cream	Butter	Blue cheese
			(mM/100 g. fat)		
0.09	0.16	0.28	0.17	0.12	0.47
0.07	0.16	0.23	1.13[a]	0.09	0.44
0.06	0.18	0.15	0.08	0.10	0.34
0.07	0.17	0.19	0.15	0.18	0.13
0.06	0.15	0.18	0.10	0.14	0.39
0.09	0.14		0.09	0.12	
0.10	0.16		0.09	0.16	
0.05	0.13		0.15	0.19	
0.09	0.19			0.29	
0.06	0.06			0.25	
0.07	0.10			0.78	
0.12				0.11	
0.08				0.19	
0.07				0.12	
0.09				0.18	
				0.17	
				0.12	
				0.12	
				0.16	
Av. 0.078	0.145	0.206	0.119	0.189	0.354

[a] Omitted from the average because of age and a rancid flavor.

the milk as obtained from the udder. Further evidence in support of this premise is the removal of the monoglycerides by saponification. The small amount present indicates either that some lipolysis had occurred previous to sampling or to extraction or that fat synthesis was incomplete. The amounts of monoglyceride in the fresh raw samples represent the minimum concentrations that can be estimated with accuracy using the method, since if more than 25% fat is dissolved in chloroform for the analysis, a single-phase system can not be maintained during the test. The accuracy at the 0.1 m*M* level is high, because added monoglycerides at this level were recovered quantitatively (4). By comparison, very rancid milk may contain up to 2.0 m*M* of monoglyceride per 100 g. of fat.

The pasteurized whole and homogenized milk samples were not related to the fresh whole samples or to each other. They were taken from the Department plant after processing, bottling, and cold storage. The larger quantities of monoglycerides found in these products are undoubtedly the result of lipolysis which occurred prior to pasteurization. Almost all the milk used by the Department is obtained with the use of pipeline milkers and bulk tanks and is subject to the usual delays. Thus, there were many opportunities for lipolysis to occur. The milk in these samples was from one to four days old when analyzed. Since 0.2 m*M* of monoglyceride added to milk caused a reduction in surface tension (2), some of the variations in surface tension at constant temperature noted by Watson (7) may have been due to the presence of monoglycerides in the samples.

The heavy cream samples were taken from a bulk supply of unknown age and history and from freshly separated and pasteurized cream. They have no relationship to the other samples. The sample with the relatively high monoglyceride content came from the former source. Since this sample was rancid organoleptically and was at least six days old when sampled, it was omitted when the heavy cream average was obtained. It is possible that puzzling variations in the whippability of heavy cream may be due to varying concentrations of monoglycerides, as shown in Table 1. However, a few preliminary trials indicated little relationship between creams of near-average monoglyceride content and whipping time. It is evident that this facet needs further exploration, perhaps utilizing additions of purified commercial monoglycerides.

Three samples of butter contained relatively large amounts of monoglyceride. These samples were given low flavor scores due to a pronounced old-cream flavor defect. It is possible that the test for monoglycerides could be used for the detection of butter made from lipolyzed cream in a manner similar to the WIA test. Diacetyl and 2,3-butylene glycol, which may be present in butter, do not interfere (3). Monoglycerides in cream undoubtedly influence churning and probably the body of butter. They are added to margarine as emulsifiers to prevent leakiness and should have the same effect in butter.

Mold-ripened cheese contained comparatively large amounts of monoglyceride, which may have some influence on the flavor of Blue and similar cheeses.

It appears that milk and milk products may contain monoglycerides that are most likely the result of lipolysis. Since monoglycerides are surface-active,

some of the variations in surface and interfacial properties of milk may be explained in terms of these compounds (1, 2).

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INHIBITION OF CERTAIN TYPES OF BACTERIAL SPOILAGE IN CREAMED COTTAGE CHEESE BY THE USE OF A CREAMING MIXTURE PREPARED WITH *STREPTOCOCCUS CITROVORUS* [1]

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SUMMARY

A special creaming mixture consisting of 1.5 parts of cream containing 20% fat and 1.0 part of an acidified culture of *Streptococcus citrovorus (Leuconostoc citrovorum),* by weight, prevented slime formation on creamed cottage cheese inoculated with *Pseudomonas fragi* or *Pseudomonas putrefaciens.* The biacetyl content of regularly creamed cottage cheese was decreased by *P. fragi* and *P. putrefaciens,* but these organisms did not decrease the biacetyl content of the specially creamed cottage cheese. Coliform organisms also were inhibited in cottage cheese creamed with the special creaming mixture, but *Geotrichum candidum* and *Candida pseudotropicalis* were not inhibited.

The addition of acetic or propionic acid to creamed cottage cheese delayed the formation of slime by *P. fragi,* and the delay was related to the amount of acid added. However, relatively large amounts of acetic or propionic acid did not entirely eliminate slime formation and the addition of these acids was not as effective as the special creaming mixture. Therefore, the volatile acids formed by *S. citrovorus* in the special creaming mixture are not considered to be the cause of inhibition of *P. fragi.*

Cottage cheese creamed with the special creaming mixture always had a slightly lower pH than cheese creamed with a regular creaming mixture. Adjusting the pH of the regular creaming mixture to the same pH as the special creaming mixture (pH 5.2) did not delay the rate of slime formation by *P. fragi.* Therefore, some factor other than the pH was responsible for the inhibition.

Creamed cottage cheese is a rather perishable product because of its ability to support the growth of many microorganisms under the usual conditions of storage. Those microorganisms considered as contaminants, and which gain access to the pasteurized skimmilk used in manufacture or to the curd during manufacture, creaming, and packaging, are an important cause of deterioration.

Considerable research has been conducted for improving the keeping quality of creamed cottage cheese. The microorganisms associated with certain defects have been classified (3, 4, 11) and the effect of pH (2, 4, 11), salt (2, 11), and temperature (2, 4, 6) on their rate of growth has been determined. Since organisms capable of causing spoilage may be derived from equipment (9, 14) or the water supply (6, 11), studies have been conducted on the elimination of them from these sources by application of bactericides (2, 5, 14).

Mather and Babel (10) developed a method for standardizing the biacetyl content of creamed cottage cheese. Experiments with this method indicated that cottage cheese creamed with a creaming mixture prepared with *Streptococcus citrovorus (Leuconostoc citrovorum)* had better keeping quality than cottage

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cheese creamed in the regular manner. The study reported here was conducted to substantiate this observation.

METHODS

Biacetyl and acetylmethylcarbinol. The colorimetric method of Prill and Hammer (12) was used for determining biacetyl and also acetylmethylcarbinol. Color intensity was measured with a Beckman spectrophotometer (Model B) at a wave length of 430 mμ. Dimethylglyoximate was used in constructing the standard curve.

Determination of pH. Cheese samples were prepared and tested by the method of Sanders (13). A Leeds and Northrup Type K potentiometer fitted with a quinhydrone electrode and saturated calomel cell was used for all pH measurements on cheese. A Beckman Laboratory Model G pH meter was used to adjust the pH of the special creaming mixtures.

Preparation of creaming mixture. The regular creaming mixture was standardized to contain 12.0% fat and was added to cottage cheese at the rate of one part creaming mixture to two parts of cottage cheese. The special creaming mixture was prepared by the method of Mather and Babel (10), using cream containing 20% fat and an acidified skimmilk culture of *S. citrovorus*; it also contained 12.0% fat and was added to cottage cheese at the same rate as the regular creaming mixture.

Source of cottage cheese. Some of the cottage cheese was manufactured by the long-set method outlined by Hales (8). However, since the study involved the effect of the method of creaming on keeping quality, cottage cheese manufactured in several commercial plants also was used in the experiments.

Source of cultures. The cultures of *Pseudomonas fragi* and *Pseudomonas putrefaciens* were recent isolations from slimy cottage cheese. All of the other cultures were from the Purdue Dairy Department collection.

Enumeration of microorganisms. Counts on suspensions of *P. fragi* and *P. putrefaciens* were made on plate count agar; the plates were incubated at 21° C. for five days. Cottage cheese and creamed cottage cheese samples for yeast, mold, and coliform counts were treated as follows: The entire contents of a 12-oz. carton of cheese were transferred to a sterile 600-ml. beaker and mixed thoroughly with a sterile spatula. Fifty grams of the mixed sample and 50 ml. of sterile sodium citrate solution (2%) were placed in a Waring blendor (previously sterilized with a chlorine solution containing 600 p.p.m. available chlorine for 3 to 5 min. and subsequently rinsed with sterile distilled water) and mixed for about 1 min. Portions of the mixture were plated directly or dilutions were prepared. Coliform counts were made with violet red bile agar and plates incubated according to *Standard Methods* (1). Yeast and mold counts were made with potato dextrose agar acidified to pH 3.5; the plates were incubated at 21° C. for five days.

RESULTS

Effect of a special creaming mixture prepared with S. citrovorus on the biacetyl content and keeping quality of creamed cottage cheese inoculated with

TABLE 1

Biacetyl, acetylmethylcarbinol, and pH values of cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese, without and with additions of *Pseudomonas fragi* to the creaming mixtures

Lot No.[a]	Days held at 45° F.														
	0	2	5	7	12	0	2	5	7	12	0	2	5	7	12
	Biacetyl					Acetylmethylcarbinol					pH				
	(p.p.m.)														
1	0.5	0.5	0.3	0.3	0.3	8.1	8.0	8.5	7.3	6.7	4.57	4.70	4.70	4.59	4.71
2	0.3	0.4	0.2	0.3	0.3	6.0	5.4	5.9	6.2	5.7	4.95	5.01	5.03	5.01	5.04
3	0.3	0.3	0.0	....	....	6.0	6.2	3.5	....	....	4.95	5.10	5.08	.....	.....
4	1.6	2.4	2.4	2.5	2.6	41.4	51.2	46.6	57.0	59.0	4.87	4.94	4.90	4.89	4.90
5	1.6	2.2	2.4	2.4	2.5	40.4	49.0	46.6	56.7	55.6	4.82	4.92	4.90	4.92	4.92

[a] Lot 1. Cottage cheese; Lot 2. Regularly creamed cottage cheese; Lot 3. Regularly creamed cottage cheese plus *P. fragi*. The creamed cottage cheese contained 67,000 *P. fragi* organisms per gram. A fruity flavor and slime were evident at seven days; Lot 4. Creamed with a special creaming mixture prepared with *Streptococcus citrovorus*; Lot 5. Same as Lot 4, except that *P. fragi* was added. The creamed cheese contained 67,000 *P. fragi* organisms per gram. A fruity flavor or slime was not evident at 12 days.

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P. fragi. To determine whether a special creaming mixture influenced the keeping quality of creamed cottage cheese, *P. fragi* was selected as one of the test organisms, since it is a common cause of fruity flavor and surface slime. A quantity of cottage cheese was taken from one vat and treated as follows: Lot 1, no treatment; Lot 2, creamed with a regular creaming mixture; Lot 3, same as Lot 2 except that *P. fragi* was added in an amount equivalent to 67,000 organisms per gram of cheese; Lot 4, creamed with a special creaming mixture; and Lot 5, same as Lot 4 except that *P. fragi* was added at the rate used in Lot 3. The results of a representative trial are given in Table 1.

The biacetyl and acetylmethylcarbinol contents of cottage cheese and regularly creamed cottage cheese changed only slightly during storage at 45° F. for 12 days. However, when *P.* fragi was added to regularly creamed cottage cheese and held for five days, biacetyl was not detectable, the carbinol content decreased from 6.0 to 3.5 p.p.m., and the pH increased from 4.95 to 5.08; a fruity flavor and slime were evident at seven days. Cottage cheese creamed with the special creaming mixture prepared with *S. citrovorus* increased in biacetyl and carbinol throughout the 12-day holding period and the increase in the cheese with *P. fragi* added was approximately the same as in the cheese without such an addition. No evidence of slime or fruity flavor was noted in the cheese creamed with the special creaming mixture at 12 days.

Growth of coliform organisms in cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese. Since coliform organisms are considered undesirable in any dairy product and frequently produce unclean flavors and other defects, experiments were conducted to determine their rate of growth in cottage cheese and creamed cottage cheese. The cottage cheese selected for this study was obtained from a plant which regularly had some coliform organisms in its cottage cheese as a result of contamination from equipment and perhaps some growth during manufacture. One portion of cottage cheese was divided into five lots and treated as follows: Lot 1, no treatment; Lot 2, creamed with a regular creaming mixture; Lot 3, same as Lot 2 except that it received an addition of *Escherichia coli;* Lot 4, creamed with a special creaming mixture prepared with *S. citrovorus*; and Lot 5, same as Lot 4 except that it received the same amount of *E. coli* as Lot 3. Data from a typical trial are presented in Table 2.

The cottage cheese used in this trial contained ten coliform organisms per gram and the population remained quite constant for six days at 45° F. Further holding showed a decrease in coliform organisms. Considerable growth of coliforms occurred in regularly creamed cottage cheese (Lots 2 and 3), regardless of the initial coliform population. Lot 2 increased in coliforms from 20 to 8,000 per gram, and Lot 3 from 1,600 to 740,000 per gram during 13 days at 45° F. One lot of cheese (Lot 4) creamed with the special creaming mixture decreased in coliform count from eight to less than two per gram in six days, whereas Lot 5 increased from 1,800 to 3,400 per gram during the 13-day holding period.

Effect of addition of a special creaming mixture to cottage cheese on the rate of spoilage by P. putrefaciens. A culture of *P. putrefaciens* isolated from a sample of spoiled creamed cottage cheese, and which produced slime and a putrid

TABLE 2

Rate of growth of coliform organisms in cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese held at 45° F.

Lot No.[a]	Days held at 45° F.									
	0	3	6	10	13	0	3	6	10	13
	Coliform count per gram					pH				
1	10	12	10	<2	<2	4.54	4.52	4.51	4.51	4.53
2	20	260	290	3,800	8,000	4.92	4.96	4.95	4.88	4.94
3	1,600	13,000	140,000	260,000	740,000	4.90	4.93	4.94	4.92	4.95
4	8	22	<2	<2	<2	4.79	4.80	4.82	4.89	4.76
5	1,800	2,400	3,000	3,800	3,400	4.78	4.84	4.83	4.81	4.77

[a] Lot 1. Cottage cheese; Lot 2. Regularly creamed cottage cheese; Lot 3. Regularly creamed cottage cheese plus addition of *Escherichia coli;* Lot 4. Creamed with a special creaming mixture prepared with *Streptococcus citrovorus;* Lot 5. Same as Lot 4, except that *E. coli* was added.

odor, was used to study the effect of the type of creaming mixture on the rate of spoilage. The design of this experiment was the same as that employed to study the rate of spoilage by *P. fragi* (Table 1). Results of a representative trial in which *P. putrefaciens* was used as the spoilage organism are given in Table 3.

The cottage cheese (Lot 1) used in this trial contained 0.4 p.p.m. biacetyl and had a pH of 4.6. No appreciable change occurred in the biacetyl content or pH during holding at 45° F. for 14 days and no slime or off-flavor developed. When creamed with a regular creaming mixture (Lot 2), the biacetyl content was decreased to 0.3 p.p.m. and the pH was increased to 4.95. This lot also showed no appreciable change in biacetyl content or pH during storage. The addition of *P. putrefaciens* to regularly creamed cheese in an amount equivalent to 1,100 organisms per gram of creamed cheese resulted in the appearance of slime in six days with storage at 45° F.; no biacetyl could be detected on the tenth day of storage. Cheese creamed with the special creaming mixture, either without (Lot 4) or with (Lot 5) an addition of *P. putrefaciens*, did not show any detectable spoilage during holding at 45° F. for 14 days. Also, Lots 4 and 5 contained more biacetyl after storage for 14 days than was present initially.

Growth of molds and yeasts in cottage cheese creamed with a regular and special creaming mixture. After experiments had indicated that the creaming mixture prevented slime formation due to *P. fragi* and *P. putrefaciens,* and also decreased the rate of growth of coliform organisms, trials were conducted to determine whether the special creaming mixture would decrease the rate of growth of molds and yeasts. For this experiment, a quantity of cottage cheese was divided into five lots and treated as follows: Lot 1, no treatment; Lot 2, creamed with a regular creaming mixture; Lot 3, same as Lot 2, except that a culture of *Geotrichum candidum* was added; Lot 4, creamed with a special creaming mixture; and Lot 5, same as Lot 4 except that *G. candidum* was added at the rate used in Lot 3. All of the lots were held at 45° F. and plated for molds at various intervals. Data obtained in a typical trial are given in Table 4.

The uncreamed cheese (Lot 1) contained four molds per gram initially and 80 per gram after holding at 45° F. for ten days. Regularly creamed cheese (Lot 2) contained two molds per gram initially and 74,000 per gram after ten

TABLE 3

Rate of slime formation, biacetyl content, and pH values of cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese, without and with additions of *Pseudomonas putrefaciens* to the creaming mixtures

Lot No.[a]	Days held at 45° F.														
	0	3	6	10	14	0	3	6	10	14	0	3	6	10	14
	Slime formation[b]					Biacetyl (p.p.m.)					pH				
1	—	—	—	—	—	0.4	0.4	0.4	0.4	0.3	4.60	4.61	4.61	4.62	4.63
2	—	—	—	—	—	0.3	0.3	0.4	0.4	0.3	4.95	4.94	4.95	4.97	4.96
3	—	—	+	+	+	0.3	0.3	0.2	0.0	0.0	4.96	4.95	4.97	4.99	4.92
4	—	—	—	—	—	2.0	2.3	2.3	2.5	2.8	4.88	4.91	4.92	4.92	4.90
5	—	—	—	—	—	2.0	2.2	2.4	2.6	2.6	4.86	4.90	4.94	4.90	4.90

[a] Lot 1. Cottage cheese; Lot 2. Regularly creamed cottage cheese; Lot 3. Regularly creamed cottage cheese plus *P. putrefaciens.* The creamed cottage cheese contained 1,100 *P. putrefaciens* organisms per gram; Lot 4. Creamed with a special creaming mixture containing *Streptococcus citrovorus;* Lot 5. Same as Lot 4, except that *P. putrefaciens* was added. The creamed cottage cheese contained 1,100 *P. putrefaciens* organisms per gram.

[b] — = slime absent, + = slime present.

TABLE 4

Growth of *Geotrichum candidum* in cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese held at 45° F.

Lot No.[a]	Days held at 45° F.							
	0	3	6	10	0	3	6	10
	Molds per gram				pH			
1	4	6	12	80	4.59	4.59	4.62	4.59
2	2	<2	34	74,000	5.00	4.99	5.02	5.00
3	240	280	2,400	3,000,000	5.01	5.02	5.04	5.05
4	<2	<2	<2	38	4.87	4.89	4.88	4.87
5	220	260	1,000	4,500,000	4.85	4.87	5.00	5.02

[a] Lot 1. Cottage cheese; Lot 2. Regularly creamed cottage cheese; Lot 3. Regularly creamed cottage cheese plus *G. candidum;* Lot 4. Creamed with a special creaming mixture containing *Streptococcus citrovorus;* Lot 5. Same as Lot 4, except that *G. candidum* was added.

days. Lot 3, to which *G. candidum* was added, had an initial count of 240 per gram and after ten days the count increased to 3,000,000 per gram. Cheese creamed with the special creaming mixture (Lot 4) contained less than two molds per gram initially and 38 per gram after holding for ten days. The addition of *G. candidum* to cheese creamed with the special creaming mixture (Lot 5) resulted in an initial count of 220 per gram and the count increased to 4,500,000 per gram in ten days.

Experiments also were conducted to determine the rate of growth of *Candida pseudotropicalis* in regularly creamed and specially creamed cottage cheese. Data obtained in these experiments are not presented, since they indicate that the type of creaming mixture had no influence on the rate of multiplication of this organism.

Effect of addition of acetic or propionic acid to creamed cottage cheese on the rate of formation of slime and fruity flavor by P. fragi. During the fermentation of citric acid by *S. citrovorus,* appreciable amounts of volatile acids (acetic and propionic) are formed. It seemed possible that these acids might be present in the special creaming mixture in sufficient amount to prevent slime and fruity flavor due to *P. fragi.* Therefore, various amounts of acetic or propionic acid were added to regularly creamed cottage cheese contaminated with *P. fragi.* For comparative purposes, two lots of specially creamed cottage cheese were included in the study; one without and one with an addition of *P. fragi.*

Data presented in Table 5 show the amounts of acetic or propionic acid added to 12-oz. cartons of creamed cottage cheese and the time required for development of slime and fruity flavor when held at 45° F. Regularly creamed cheese, with no additions, showed surface slime in ten days and a fruity flavor in nine days; whereas, cheese with an addition of *P. fragi* developed these defects in seven and five days, respectively. The addition of either acetic or propionic acid increased the time required for development of slime and fruity flavor, and the increase was related to the amount of acid added. However, the largest amount of acetic or propionic acid added to regularly creamed cottage cheese did not delay slime formation or a fruity flavor for as long a time as the special creaming mixture.

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TABLE 5

Rate of development of slime and fruity flavor in creamed cottage cheese without and with additions of *Pseudomonas fragi* and acetic or propionic acid

Product and treatment	Number of days sample was held at 45° F. before detection of	
	Slime	Fruity flavor
Regularly creamed cottage cheese	10	9
Regularly creamed cottage cheese plus *P. fragi*	7	5
Regularly creamed cottage cheese plus *P. fragi* and 1 ml. of 0.2 *N* acetic acid	9	8
Regularly creamed cottage cheese plus *P. fragi* and 3 ml. of 0.2 *N* acetic acid	10	9
Regularly creamed cottage cheese plus *P. fragi* and 5 ml. of 0.2 *N* acetic acid	11	10
Regularly creamed cottage cheese plus *P. fragi* and 1 ml. of 0.2 *N* propionic acid	7	6
Regularly creamed cottage cheese plus *P. fragi* and 3 ml. of 0.2 *N* propionic acid	12	10
Regularly creamed cottage cheese plus *P. fragi* and 5 ml. of 0.2 *N* propionic acid	12	10
Regularly creamed cottage cheese plus *P. fragi* and 7 ml. of 0.2 *N* propionic acid	13	11
Cottage cheese creamed with a special creaming mixture containing *S. citrovorus*	>15	>15
Cottage cheese creamed with a special creaming mixture containing *S. citrovorus* plus an addition of *P. fragi*	>15	>15

Effect of pH of creaming mixture on the rate of slime formation by P. fragi. The special creaming mixture prepared with *S. citrovorus* had a pH of about 5.2 when added to cottage cheese. Therefore, the pH of cheese creamed with the special creaming mixture was always slightly lower than the pH of regularly creamed cottage cheese. To determine whether the lower pH was inhibitory to *P. fragi,* a regular creaming mixture was acidified with citric acid to pH 5.2 and added to cottage cheese. A comparison of the rate of slime formation by *P. fragi* on cottage cheese, regularly creamed cottage cheese, cottage cheese creamed with an acidified creaming mixture, and cottage cheese creamed with the special creaming mixture is shown in Table 6.

The cottage cheese used in this experiment had an initial pH of 4.80 and creaming with a regular creaming mixture increased the pH to 5.19. No slime was formed on the cottage cheese during holding at 45° F. for 13 days, but slime was noted on the regularly creamed cheese at 13 days. The addition of *P. fragi* to regularly creamed cottage cheese caused slime to appear in five days. Cottage cheese creamed with the mixture acidified with citric acid to pH 5.2 had an initial pH of 5.01, and this cheese also developed surface slime in five days. Cheese creamed with the special creaming mixture prepared with *S. citrovorus* had an initial pH of 5.08 and did not show surface slime in 13 days, even when *P. fragi* was added to the creaming mixture.

DISCUSSION

During the development of a method for increasing and standardizing the biacetyl content of creamed cottage cheese (10), it was realized that some microorganisms capable of causing spoilage might interfere with such a procedure.

TABLE 6

Rate of slime formation on cottage cheese creamed with a regular creaming mixture, a regular creaming mixture acidified with citric acid, and a special creaming mixture prepared with *Streptococcus citrovorus*

	Days held at 45° F.							
	0	5	9	13	0	5	9	13
Lot No.[a]	Slime present (+) or absent (−)				pH			
1	−	−	−	−	4.80	4.78	4.77	4.82
2	−	−	−	+	5.19	5.15	5.13	5.14
3	−	+	+	+	5.18	5.13	5.18	5.16
4	−	+	+	+	5.01	5.00	5.02	5.00
5	−	−	−	−	5.08	5.05	5.05	5.05
6	−	−	−	−	5.07	5.07	5.03	5.05

[a] Lot 1. Cottage cheese; Lot 2. Regularly creamed cottage cheese; Lot 3. Regularly creamed cottage cheese plus *Pseudomonas fragi;* Lot 4. Cottage cheese creamed with acidified creaming mixture plus *P. fragi;* Lot 5. Specially creamed cottage cheese; Lot 6. Specially creamed cottage cheese plus *P. fragi.*

Earlier studies by Parker *et al.* (11) and Wales and Harmon (15) indicated that certain spoilage organisms, particularly *Pseudomonas* species, could decrease the biacetyl content of creamed cottage cheese. However, as the experimental work progressed, it was noted that the cottage cheese creamed with a special creaming mixture prepared with *S. citrovorus* did not develop certain defects as readily as regularly creamed cheese. The defect noted most frequently in the regularly creamed cheese was a fruity odor and surface slime accompanied by loss of typical flavor. The causative organism was *P. fragi.*

Controlled experiments were conducted to determine whether the special creaming mixture actually was inhibiting slime formation due to *P. fragi* and also to study the effect of the organism on the biacetyl content. The data presented in Table 1, as well as additional experiments which are not reported, confirm the earlier observations and indicate that *P. fragi* does decrease the biacetyl content of regularly creamed cheese but has no effect on the biacetyl content of cheese creamed with the special creaming mixture. The absence of slime on the specially creamed cheese, even with rather large numbers (67,000 per gram) of *P. fragi* added, indicates the value of the special creaming mixture in preventing defects due to this common spoilage organism.

It was of interest to determine what effect the special creaming mixture would have on other microorganisms capable of causing defects in creamed cottage cheese. Therefore, *E. coli, P. putrefaciens, G. candidum,* and *C. pseudotropicalis* were chosen for additional studies.

E. coli, as well as other coliform organisms present in the creamed cottage cheese as a result of contamination, were inhibited by the special creaming mixture (Table 2). Coliform counts on samples of cottage cheese creamed with the special creaming mixture in commercial plants also indicated that this creaming mixture decreased the rate of growth of coliform organisms.

Inhibition of *P. putrefaciens* by the use of the special creaming mixture was expected, because of the results obtained with *P. fragi* and certain similarities among the two organisms. Controlled experiments (Table 3) confirmed such an inhibition.

The special creaming mixture had no effect on the growth of *G. candidum* or *C. pseudotropicalis*. Other molds and yeasts were not included in this study.

Gelpi and Rusoff (7) indicated that the addition of propionic acid prolonged the shelf-life of creamed cottage cheese. Since the special creaming mixture contained some propionic and acetic acid as a result of the fermentation of citric acid by *S. citrovorus*, experiments were conducted to determine the extent of inhibition of *P. fragi* by these acids. The data presented in Table 5 show that the addition of acetic or propionic acid to creamed cottage cheese, in amounts greater than those present in the special creaming mixture (10), did not inhibit slime formation for as long a period as the special creaming mixture.

The pH of cottage cheese creamed with the special creaming mixture was always slightly lower than the pH of cottage cheese creamed with a regular creaming mixture. Several investigators (2, 4, 11) have indicated that the pH of creamed cottage cheese influences the rate of slime formation by bacteria. Therefore, experiments were conducted to determine whether it was the pH or some other factor which inhibited slime formation due to *P. fragi* or *P. putrefaciens* and also inhibited the growth of coliform organisms. The data presented in Table 6, as well as other trials not reported here, indicate that the lower pH of the special creaming mixture is not entirely responsible for the inhibition or prevention of defects by *P. fragi*.

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VITAMIN B_{12} IN WHEYS PREPARED BY COAGULATING MILK WITH ACID AND RENNET[1]

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SUMMARY

The U.S.P. microbiological assay procedure for vitamin B_{12} activity was followed, using *Lactobacillus leichmanii* ATCC 7830. Three samples of pasteurized milk and 16 samples of pasteurized skimmilk averaged, respectively, 4.0 γ and 4.7 γ of vitamin B_{12} per liter. Comparison of amounts of vitamin B_{12} in wheys and original milks indicated that both casein and whey proteins bind the vitamin. Rennet extract caused hydrolysis and/or separation from casein of some of the bound vitamin B_{12}. Six rennet wheys contained an average of 60% of the vitamin B_{12} present in the original milk or skimmilk. Six acid wheys contained an average of 44% of the vitamin present in the original skimmilk. The separation of bound vitamin B_{12} from casein was influenced by rennet concentration and time at 40° C.

Gregory and coworkers (2, 3) found no vitamin B_{12} in ultrafiltrates of milks from several animal species, and concluded that it is bound by milk proteins. Both casein and whey proteins are reported as good sources of B_{12} (6). Cottage cheese, by *Lactobacillus leichmanii* assay, contains an average of 8.8 γ of vitamin B_{12} per kilogram, as compared to 10.3 γ per kilogram for Cheddar cheese (5). Pasteurized milk contains 3.4 γ to 4.4 γ per liter (1). Comparison of these values with average yields of cheese suggests that the retention of vitamin B_{12} by casein is greater in cottage cheese making than in Cheddar cheese making.

This paper reports on a study of the distribution of vitamin B_{12} in wheys prepared by coagulating milk with acid and rennet.

METHODS

Vitamin assay. The U.S.P. microbiological assay procedure for vitamin B_{12} activity (7), with revised medium (8), was followed, using *Lactobacillus leichmanii* ATCC 7830. One milliliter of each material to be tested was diluted with 10 ml. of sterile distilled water, adjusted to pH 6.0, and diluted to desired volume. Inoculated culture tubes of the assay medium were incubated for 72 hr. at 32° C. Bacterial growth responses were measured by electrometric titration to pH 7.0 with 0.1 *N* sodium hydroxide. The standard vitamin B_{12} solution was made from crystalline vitamin B_{12}.

Acid coagulation. Portions of 100 ml. of skimmilk were adjusted electrometrically to pH 4.6 with glacial acetic acid (dispersed with a micropipette at 35° C.). Less than 0.5 ml. of acid was required. Whey was separated from the casein fraction by filtration through coarse filter paper.

Rennet coagulation. Portions of 100 ml. of skimmilk were adjusted to and maintained at 40° C. in a thermostatically controlled water bath. Unless indi-

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[1] These data were taken in part from a thesis presented by the junior author in partial fulfillment of the requirements for the degree of Master of Science, University of California.

cated otherwise, the following procedure was used: Each portion received 0.5 ml. of rennet extract. After rennet was added, samples were mixed, permitted to stand 15 min., cut into cubes with a stainless-steel spatula, kept at 40° C. for an additional 30 min., and filtered through coarse filter paper.

RESULTS

Experimental error. The experimental error of the assay procedure was determined by assaying in duplicate four samples of pasteurized skimmilk and four samples of whey. The average vitamin B_{12} contents were 4.25 γ and 2.88 γ per liter for skimmilk and whey, respectively. For duplicate determinations on skimmilk, the average deviation from the mean was 0.13 γ per liter (range 0.08 to 0.17 γ). For duplicate determinations on whey, the average deviation from the mean was 0.05 γ per liter (range 0.04 γ to 0.06 γ).

Vitamin B_{12} in milk and skimmilk. Three samples of pasteurized milk and 16 samples of pasteurized skimmilk were assayed for vitamin B_{12} content during the study. The samples of milk averaged 4.0 γ of vitamin B_{12} per liter (range 3.5 to 4.8 γ). The samples of skimmilk averaged 4.7 γ per liter (range 3.5 to 6.6 γ).

Vitamin B_{12} in acid and rennet wheys. Wheys were prepared from a sample of pasteurized skimmilk by coagulation with acid and rennet. The total solids contents of the wheys were not appreciably different (6.78% for the acid whey; 6.88% for the rennet whey), but the acid whey contained only 1.9 γ of vitamin B_{12} per liter, as compared to 2.8 γ per liter for the rennet whey. These amounts were 44 and 65% of the amount of vitamin B_{12} present in the original skimmilk (4.3 γ per liter).

A sample of the rennet whey was adjusted to pH 4.6 and filtered. This procedure did not change its vitamin B_{12} content. But one sample of acid whey, prepared by adjusting skimmilk below the isoelectric point of casein to pH 4.0 (to permit some of the casein to redissolve before filtering), contained 2.6 γ of B_{12} per liter.

The above difference in vitamin content of acid and rennet wheys was substantiated by vitamin B_{12} assays for two additional acid wheys, three cottage cheese wheys, three additional rennet wheys, and two Cheddar cheese wheys (Table 1).

Influence of rennet concentration and time on the vitamin B_{12} content of whey. Since the vitamin B_{12} content of rennet whey was higher than that of acid whey, different concentrations of rennet were used for coagulating portions of skimmilk, and different periods of time at 40° C. were permitted after the rennet addition before filtration. The results (Table 2) show that the vitamin B_{12} content of whey increased with time at 40° C. and with rennet concentration.

DISCUSSION

Gregory and coworkers (2, 3) found that vitamin B_{12} in milk is bound by milk proteins. Comparisons of the amounts of Vitamin B_{12} in wheys and original milks reported in this paper show that both casein and whey proteins bind the

TABLE 1

Vitamin B_{12} in wheys prepared by coagulating pasteurized skimmilk or milk with acid and rennet

Sample	Vitamin B_{12} content		
	(1) Skimmilk or milk	(2) Whey	$\frac{(2)}{(1)} \times 100$
	(γ/liter)	*(γ/liter)*	*(%)*
Acid preparation			
1A	4.3	1.9	44
2A	4.7	2.3	49
3	5.2	2.1	40
4[a]	4.4	2.1	46
5[a]	5.6	2.6	46
6[a]	3.5	1.4	40
Average			44
Rennet preparation			
1B	4.3	2.8	65
2B	4.7	3.2	68
7	5.5	3.3	60
8	5.8	3.1	52
9[b]	3.5[c]	1.9	54
10[b]	3.7[c]	2.4	65
Average			60

[a] Whey taken as drained from Cottage cheese curd.
[b] Whey taken as drained from Cheddar cheese curd.
[c] Milk.

vitamin. Acid and rennet wheys contained much smaller amounts of B_{12} than did the milk or skimmilk from which they were prepared. The decreases resulted from precipitation of vitamin B_{12} with casein and not from inactivation of the vitamin, as is evident from two results: (1) The vitamin B_{12} content of rennet whey was not decreased by adjustment to pH 4.6 and filtration. (2) Acid whey prepared from skimmilk adjusted below the isoelectric point to pH 4.0 contained more vitamin B_{12} than did whey prepared from a duplicate sample of skimmilk adjusted to pH 4.6. The conclusion that both casein and whey proteins bind the vitamin is in agreement with an earlier study in which both crude casein and dried whey proteins were found to be good sources of vitamin B_{12} (6).

The results indicate that rennet hydrolyzes and/or separates bound vitamin B_{12} from casein. This action possibly is the same as that of papain or trypain used to make the vitamin B_{12} in milk available to microorganisms (2). The separation is influenced by rennet concentration and time at 40° C. Undoubtedly, other factors, such as temperature and pH, are important. In an experiment not reported above, rennet extract was separated into rennin and pepsin fractions by the method of Hankinson and Palmer (4). Results with the fractions indicated that rennin and pepsin serve equally well in separating vitamin B_{12} from casein, but it is not certain that complete separation of rennin and pepsin was achieved.

The microbiological assay values reported by Hartman *et al.* (5) for vitamin B_{12} in cheese are consistent with finding that rennet separates vitamin B_{12} from casein. They found cottage cheese and Cheddar cheese to contain 8.8 and 10.3 γ of B_{12} per kilogram, respectively. Using these values, and assuming that the milk, cottage cheese, and Cheddar cheese contained 3.3, 15, and 32% protein,

TABLE 2

Influence of rennet concentration and time at 40° C. on the vitamin B_{12} content of whey

Rennet per 100 ml. of skimmilk	Time from adding rennet to filtering	Vitamin B_{12} content of whey[a] Results for Trial No.:				Average
		1	2	3	4	
(ml.)	*(hr.)*	*(%)*[b]	*(%)*	*(%)*	*(%)*	*(%)*
0.05	0.5		52		57	54
	1.0		53		62	57
	2.0		54		64	59
0.10	0.5		54			54
	1.0		58			58
	2.0		59			59
0.5	0.75	61				61
	1.25	70				70
	3.25	76				76
1.0	0.5		65		72	68
	1.0		66		75	70
	2.0		67		75	71
1.0	0.75	76				76
	1.25	88				88
	3.25	89				89
3.0	0.5			64		64
	1.0			78		78
	2.0			82		82
5.0	0.5				78	78
	1.0				79	79
	2.0				81	81
5.0	0.75	74				74
	1.25	89				89
	3.25	92				92

[a] The vitamin B_{12} contents were corrected for volume changes due to the addition of rennet and to exclude 70% of the vitamin B_{12} present in the rennet extract. The rennet extract contained 11.6 γ of vitamin B_{12} per liter.

[b] $$\% = \frac{B_{12}\text{/liter of whey}}{B_{12}\text{/liter of skimmilk}} \times 100.$$

respectively, one finds that the respective products contained about 12, 5.6, and 3.2 γ of vitamin B_{12} per 100 g. of protein, exclusive of the B_{12} and protein contained in the cottage cheese creaming mixture.

The present finding, however, is not in agreement with a rat assay for vitamin B_{12} in cheese, reported by Hartman *et al.* (5). By rat assay they found cottage cheese and Cheddar cheese to contain 8.0 and 20.8 γ of vitamin B_{12} per kilogram, respectively, and milk to contain 7.1 γ per liter. Calculations from these values suggest that the milk, cottage cheese, and Cheddar cheese contained, respectively, 21, 4.2, and 6.5 γ of vitamin B_{12} per 100 g. of protein, exclusive of the B_{12} and protein contained in the cottage cheese creaming mixture. The ratio of these values is different from the ratio of values calculated similarly from microbiological assay results. Comparison of the ratios with regard given to the finding that rennet hydrolyzes and/or separates bound vitamin B_{12} from casein indicates error in the rat assay for vitamin B_{12} in cottage cheese. Hartman *et al.* discussed several possible reasons for disagreement between rat and microbiological assay results.

MATERIALS AND METHODS

The experiment was planned to study three consecutive corpora lutea from each of ten first-service and 11 repeat-breeder heifers. It was found, however, that abnormal ovarian activity tended to follow the removal of functional corpora lutea. Nine cystic follicles developed (three after the removal of the first corpus luteum and six after the second) and four twin ovulations occurred (three after the first, and one after the second, corpus luteum was removed). A total of 47 removals was made from the 21 heifers. Only seven first-service and seven repeat-breeder heifers produced two consecutive corpora lutea and were included in the statistical analysis.

All of the animals were purchased from Wisconsin farmers and not more than one repeat-breeder was taken from one farm. Only heifers which had been bred unsuccessfully at least four times, and whose reproductive systems appeared clinically normal upon rectal palpation, were accepted as repeat-breeders. The first-service and repeat-breeder animals were run on the experiment concurrently and all were subjected to essentially the same environment. After being placed in the experimental group, each animal was allowed to have at least one normal estrual cycle. Two consecutive corpora lutea were then removed from each heifer. The first, and in some cases the second, was removed by surgery, through a supravaginal incision, and the remainder was obtained at slaughter. All corpora lutea were obtained 14 days after estrus.

Each corpus luteum was weighed and a small portion was fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin for histological study. The luteal cells were classified into five different types, using the classification of Foley and Greenstein (1). Types I and II cells, which had vesicular nuclei, were combined, as were Types III, IV, and V, which had pyknotic and shrunken nuclei. A differential count of five microscopic fields was made for each corpus luteum and the per cent Type I plus Type II of the five types of cells was calculated. These percentages, transformed to angles, were used in the statistical analysis.

The remaining portion of each corpus luteum was stored in 95% ethanol at a subzero temperature and subsequently analyzed quantitatively for progesterone content, using the method of Loy *et al.* (3).

Corpora lutea weights, per cent Type I and II luteal cells, progesterone concentration, and total progesterone content were analyzed statistically for differences between first-service and repeat-breeder heifers and between first and second corpora lutea (the second corpus luteum being the product of an estrus following an artificially shortened cycle), and for effects of an interaction between these two. Intraclass correlations were calculated as an estimate of the within-heifer repeatability of the four variables studied. A small amount of luteal tissue was lost from one gland during removal from a first-service heifer and, consequently, data from only six heifers in this group were analyzed for variation in corpora lutea weights and total progesterone content.

RESULTS AND DISCUSSION

The ranges and averages of the weights, percentages of Type I and II luteal cells, progesterone concentrations, and total progesterone contents of the first and second corpora lutea for both first-service and repeat-breeder heifers are shown in Table 1.

The respective averages (first and second corpora lutea combined) for first-service and repeat-breeder heifers were as follows: corpora lutea, weight 4.37 and 3.77 g., per cent Type I and II cells, 77.5 and 75.7, progesterone concentration, 33.5 and 37.4 μg. per gram luteal tissue, total progesterone content 138.8 and 143.3 μg. Differences between first-service and repeat-breeder heifers were statistically nonsignificant for all four variables. No evidence was provided, therefore, that a progesterone deficiency exists in repeat-breeders at this stage of the estrual cycle. This test would not necessarily preclude the existence of a difference in the corpora lutea in which both production and release rates of progesterone were higher in one group than the other. It is assumed, however, that a difference in rate of production would be manifest by a difference in per cent of Type I and II luteal cells, and variations in rate of release only would result in a corresponding variation in gland content of the steroid. The correlation (within classes of heifers and order number of corpora lutea) between progesterone concentration and per cent Type I and II cells was found to be 0.57 ($P < 0.01$).

It may be that a subnormal progesterone level is a factor in lowered breeding efficiency but that the deficiency arises later than the 14th day. The possibility of a drop in progesterone release at the time that maintenance of the corpus luteum is shifted from mechanisms of the estrual cycle to those of pregnancy needs yet to be examined. Theoretically, an excessive drop in repeat-breeders could account for a high incidence of embryonic death between Days 16 and 25, as shown by Hawk *et al.* (2). None of the animals in the present study was bred, so a comparison between bred heifers at 14 days was not made.

The average weights of first and second corpora lutea for first-service and repeat-breeder heifers combined were 4.29 and 3.80 g., respectively. The difference between these is statistically significant. None of the remaining three variables differed significantly between first and second corpora lutea. The respective averages for first and second corpora lutea when first-service and repeat-breeder corpora lutea were combined were as follows: per cent Type I and II cells, 78.0 and 75.2, progesterone concentration, 34.7 and 36.1 μg. per gram luteal tissue, total progesterone content, 148.1 and 134.4 μg.

The repeatability within heifers of the four variables studied was estimated by intraclass correlations. This intraclass correlation was statistically significant for corpora lutea weights (0.34, $P < 0.05$), progesterone concentration (0.53, $P < 0.01$), and total progesterone content (0.53, $P < 0.01$), but was nonsignificant for per cent Type I and II cells (0.16). Whether genetics, environment, or a combination of the two is responsible for this individuality of heifers in progesterone production is not known.

TABLE 1

Ranges and averages of weight, per cent Type I and II cells, progesterone concentration, and total progesterone content of corpora lutea from first-service and repeat-breeder heifers

Heifers	Weight		Type I and II cells		Progesterone concentration		Progesterone content	
	Range	Average	Range	Average	Range	Average	Range	Average
	(g.)		(%)		(μg/g tissue)		(μg.)	
					First corpus luteum[a]			
First-service (F-S)	3.80–5.39	4.76	75–82	78.6	21.1–46.5	34.7	104–210	160.3
Repeat-breeder (R-B)	2.74–4.89	3.89	74–86	77.4	23.0–53.4	34.8	73–229	137.6
F-S and R-B	2.74–5.39	4.29	74–86	78.0	21.1–53.4	34.7	73–229	148.1
					Second corpus luteum[a]			
F-S	3.31–4.88	3.97	61–84	76.4	20.4–47.0	32.3	80–144	117.3
R-B	3.01–4.45	3.66	53–82	74.0	18.9–79.6	39.9	83–354	149.0
F-S and R-B	3.01–4.88	3.80	53–84	75.2	18.9–79.6	36.1	80–354	134.4

[a] Two consecutive corpora lutea were removed from each heifer.

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PRODUCTION AND GROWTH OF DAIRY COWS REARED ON SILAGE OR HAY RATIONS

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SUMMARY

Butterfat, milk production, and body weights of heifers reared on hay or alfalfa silage rations were compared. Those reared on silage, with resulting smaller size at 2 yr. of age, produced somewhat less butterfat and milk during the entire first lactation than did the heifers reared on hay or hay and silage. These differences were not statistically significant. At later ages, the heifers reared on silage had nearly the same body weights as those reared on hay rations.

Dairy heifers reared on wilted alfalfa silage or on this silage plus corn silage as the only forage were smaller at calving time than heifers reared on hay (8, 10). The silage-fed heifers also consumed less nutrients than did the heifers fed hay. The subsequent milk production and growth of such animals were investigated and the data are presented in this report. Results of a corollary experiment, in which the effect of restricted energy intake during the rearing period on subsequent production was examined, are also presented.

EXPERIMENTAL PROCEDURE

Details of the dietary treatments and body weights up to 2 yr. of age were presented in previous publications (8, 10). To study milk production, these animals were placed into four groups according to previous ration. Animals in Group A were reared on alfalfa hay and were composed of the heifers in Groups 1 and 4 of previous publications (8, 10). Animals in Group B were reared on alfalfa silage plus limited amounts of hay and were composed of heifers in Groups 2 and 6 of previous publications (8, 10). Animals in Group C were reared on alfalfa silage and grain and represent heifers in Group 7 of a previous publication (10). Animals in Group D were reared on silage and were composed of heifers in Groups 2C, 3, 5, and 8 of previous publications (8, 10).

During the first lactation, approximately half the heifers of each group were continued on the forage on which they were reared. The remainder were changed to alfalfa hay and corn silage at calving time. This ration also included some pasture and other roughages when the animals were over 2–3 mo. in lactation, and will be referred to as the herd ration, or Ration 1. All animals were fed Ration 1 during subsequent lactations.

In the corollary experiment, five Jersey heifers (Group E) were reared on a limited energy intake, which amounted to about 70% of that consumed by seven full-fed heifers (Group F). They were fed alfalfa hay as the only forage, plus a concentrate mix. The intake of protein, calcium, and phosphorus during the

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rearing period was equalized for both groups by feeding corn meal, corn gluten meal, and mineral mixes as necessary to Group E.

Milk and fat production was obtained from daily milk weights and butterfat tests made on a composite milk sample obtained on two consecutive days every ten days. During lactation a grain mixture was fed at the rate of 1 lb. for every 3 lb. of milk for Jersey animals and 1:4 for Holsteins. All cows were weighed three consecutive days per month or two days every ten days. Body weights at yearly intervals were calculated from all weights obtained 1 mo. previous to and 1 mo. after the yearly anniversary date without considering stage of pregnancy.

TABLE 1

Information on calving record of animals fed the different rearing rations

	A	B	C	D	E	F
	Hay	Silage + Hay	Silage + Grain	Silage		
Rearing Ration No.	1, 4	2, 6	7	2C, 3, 5, 8	Limited ration	Full ration
Rearing forage	Hay	Silage + hay	Silage + grain	Silage	Hay + grain	Hay + grain
Age at calving *(mo.)*[b]	27.7	27.1	28.1	27.7	25.4	27.3
Services/conception[b]	2.1	2.0	2.4	2.2	1.8	2.8
Discarded heifers *(%)*	11	12	8	12	0	14
Calves, stillbirths *(%)*[b]	13	0	8	8	0	0
No. born	24	35	12	51	5	6
Size *(% std.)*[a,b]	82	85	81	79	76	82
Standard deviation	±15	± 9	±15	±13	± 4	±13
No. of heifers that became prematurely dry						
Ration 1	0	0	1	3	0	0
Ration 2	1	2	2	1	0	0

[a] Birth weight calculated as per cent of standard for the respective breed and sex, References 5 and 6.

[b] No statistically significant differences between groups.

The data on milk and fat production for the first lactation only, for animals in Groups A, B, C, and D during the first 60 days and for 305 days, were analyzed statistically by fitting constants for unequal subclass numbers, as outlined by Yates (11) and Henderson (3, 4). Constants were fitted simultaneously for groups, rations, breeds, and all two-factor interactions.[1] The adjusted least-squares means are presented in Table 2. A separate one-way clasification analysis of variance was used for data on reproduction with breeds combined, but for data on body weights it was performed on data for each breed at each age indicated.

RESULTS

Information on reproductive performance and calving record is given in Table 1. These data indicate that age at calving, services per conception, percentage of heifers discarded, and size of calves apparently were not influenced by rearing ration. Heifers were discarded if they were not pregnant when they

[1] The assistance of W. R. Harvey, Biometrical Services, A.R.S., in applying these methods to these data is gratefully acknowledged.

became 2 yr. of age. The extent to which the rearing ration was responsible for this appears questionable, since there was evidence of vibrio infection at this time in the herd; however, it was not extensive enough to affect the breeding performance of cows in the herd.

There were six heifers in Groups C and D that voluntarily dried off at an early date, compared to only three in Groups A and B. This is indicated in the bottom portion of Table 1. Any effect of ration fed during lactation was not apparent.

The butterfat and milk production during the first 2 mo. of lactation was greater for the animals in Groups A and B than for those in Groups C and D ($P < 0.01$ or 0.02, Table 2). Butterfat and milk production for the 305-day period also was more for the same groups, but in only one comparison (milk—Group B > Group D) was the difference of statistical significance. These data indicate that the rearing ration, with its effect on size of heifer at parturition, had a greater influence on the level of milk and fat production in the early portion of the first lactation than on later or total production.

In comparing the production of lactating heifers fed rearing rations, as compared to heifers fed herd ration, there was little difference in the average milk or butterfat production when all groups were combined. However, in Groups C and D production was less when fed the rearing ration than when fed herd ration (1,319 and 607 lb. milk for Groups C and D, respectively). These results suggest that the relatively lower production of the C-2 and D-2 subgroups, when compared to the A-2 and B-2 subgroups, was at least partially due to the inadequacy of the ration fed during lactation. Thus, the possible carry-over effect of size of animal at parturition may have been negligible. This is indicated by the similarity in milk production for subgroups A-1, B-1, C-1, and D-1.

The significant group-breed interaction was due mainly to the different responses of the animals in Groups B and C. Holstein animals reared on silage and hay (Group B) produced slightly more than those in Group A, but the Jersey animals in Group B produced noticeably less milk and butterfat than did the Jersey animals in Group A. The Holstein animals reared on silage and grain (Group C) produced as much as those in Groups A and B, but the Jersey animals in Group C produced the least of any group.

In the corollary experiment, the milk and fat production during the first lactations of the restricted Group (E) was slightly more than that of the animals in Group F. Milk production during the first 60 days was 1,337 and 1,321 lb. and during the 305-day lactation, 6,625 and 6,543. Mean butterfat production and standard deviation for Groups E and F during the first lactation was 374 ± 41 and 373 ± 31. Similar values for the second lactation were 450 ± 78 and 394 ± 50, respectively. Certainly, the restriction of energy during the rearing period of animals in Group E did not reduce their rate of production during the first two lactations below that of heifers reared under normal feeding practices.

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TABLE 2

Least-squares means (adjusted) of butterfat and milk production during first lactation for main effects and subclasses

Classification	60-Day period			305-Day period		
	Cows	Butterfat	Milk	Cows	Butterfat	Milk
	(No.)	*(lb.)*	*(lb.)*	*(No.)*	*(lb.)*	*(lb.)*
Groups (G)—Rearing ration						
A (Hay)	23	76[1]	1,703[2]	20	301	6,580
B Silage + hay	35	74[2]	1,757[1]	31	305	6,699[1]
C Silage + grain	11	66[3]	1,503[3]	8	285	6,398
D Silage only	49	66[3]	1,517[3]	44	275	6,019[4]
Average ($\hat{\mu}$)	118	71	1,620	103	291	6,424
Rations (R)—Milking ration						
1 (Herd)	56	72	1,680	47	299	6,676
2 (Rearing ration)	62	69	1,560	56	283	6,172
Breeds (B)						
Holstein	54	77	1,963	49	301	7,732
Jersey	64	64	1,277	54	282	5,116
G-R subclasses						
A-1	7	77	1,744	5	294	6,528
A-2	16	75	1,661	15	307	6,631
B-1	18	76	1,772	16	312	6,795
B-2	17	73	1,742	15	298	6,602
C-1	4	65	1,594	3	305	7,058
C-2	7	66	1,412	5	264	5,739
D-1	27	69	1,608	23	287	6,323
D-2	22	62	1,426	21	264	5,716
G-B subclasses						
A-Holstein	12	77	1,961	10	295	7,634
A-Jersey	11	75	1,445	10	306	5,525
B-Holstein	15	83	2,170	14	319	8,324
B-Jersey	20	66	1,343	17	291	5,073
C-Holstein	5	81	1,998	5	316	8,127
C-Jersey	6	50	1,009	3	254	4,669
D-Holstein	22	68	1,722	20	272	6,841
D-Jersey	27	64	1,312	24	278	5,198
R-B subclasses						
1-Holstein	26	80	2,063	24	309	7,996
1-Jersey	30	64	1,296	23	290	5,356
2-Holstein	28	75	1,862	25	292	7,467
2-Jersey	34	64	1,259	31	275	4,877
Mean squares[a]	(df)		($\times 10^3$)	(df)		($\times 10^3$)
G	3	842*	472**	3	6,023	3,086
R	1	159	283	1	3,987	3,951
B	1	3,887**	9,968**	1	5,561	111,354**
GR	3	83	45	3	968	633
GB	3	1,407**	442*	3	4,117	4,596
RB	1	177	185	1	38	15
GRB	3	288	64	3	2,515	535
Error	102	268	118	87	3,630	1,750

[a] The least-squares given were adjusted for unequal frequencies with respect to all effects except three-factor interaction by least-squares.

$1 > 3$, $P < 0.01$; $2 > 3$, $P = 0.02$; $1 > 4$, $P < 0.05$; * = $P < 0.05$; ** = $P < 0.01$.

In general, the animals reared on silage rations (Groups C and D) produced somewhat less butterfat and milk than did those reared on hay or silage and hay (Groups A and B). However, the differences were just short of statistical significance when the entire lactation was compared, but were significant when the early portion of the lactation period was compared (Table 2). There was an indication that animals reared on silage produced more milk and butterfat during the first lactation, when the lactating ration was alfalfa hay and corn silage, than when it was alfalfa silage (C-1 and D-1 compared to C-2 and D-2, Table 2). In the corollary experiment there was an inappreciable difference in butterfat or milk production of animals that were reared to calving time on rations adequate or limited in energy. These results are in good agreement with milk production data published on animals reared on three planes of nutrition (2, 7). Similarly, rats fed on a restricted ration to parturition, thus reducing body size at this time, produced as much or more milk than females reared with an unlimited feed intake when results were based on litter growth (9).

TABLE 3

Body weight at yearly intervals of animals fed the different rearing rations

Rearing Group No.....		2	3	4	5	6	7
				Holstein females			
A	(lb.)	1,159	1,241	1,313	1,347	1,370	1,411
	(No.)	9	9	6	4	4	1
B	(lb.)	1,068	1,115	1,190	1,352	1,337	1,281
	(No.)	14	14	10	5	3	3
C	(lb.)	1,075	1,087	1,166	1,133	1,282	.....
	(No.)	5	5	4	2	1	.....
D	(lb.)	969	1,069	1,209	1,294	1,307	1,414
	(No.)	20	20	12	5	2	1
				Jersey females			
A	(lb.)	737	751	828	890	940	937
	(No.)	10	10	8	8	5	1
B	(lb.)	710	777	853	891	914	1,024
	(No.)	18	18	13	8	3	2
C	(lb.)	698	732	802	946	.....	.....
	(No.)	4	4	2	1	.....	.....
D	(lb.)	650	744	845	947	966	945
	(No.)	26	26	18	12	8	3
E	(lb.)	683	768	844	858	899	903
	(No.)	5	5	5	4	4	4
F	(lb.)	760	795	896	954	919	955
	(No.)	5	5	5	4	2	2
				Holsteins + Jerseys (as per cent of weight at 2 yr.)			
A-1		100	103	115	119	125	127
A-2		100	105	110	.....	.....	.....
B-1		100	107	117	125	127	128
B-2		100	108	113	.....	.....	.....
C-1		100	112	119	117	117	.....
C-2		100	98	105	.....	.....	.....
D-1		100	112	125	136	138	135
D-2		100	113	123	.....	.....	.....
E-1		100	112	124	123	129	129
F-1		100	105	118	131	133	138
Normal[a]		100	116	123	125	129	133

[a] References (5) and (6).

The average body weight at yearly ages of the animals is given in Table 3. The differences in body weight among the four Holstein groups or the six Jersey groups were not statistically significant at 4, 5, or 6 yr. of age. At 5 or 6 yr. of age the body weight of Holstein animals in Group D was still slightly less than the weight of animals in Groups A, B, or C. Jersey animals in Groups A, B, C, D, and F all had approximately the same body weights at 5, 6, and 7 yr. of age, but those in Group E were slightly lower at these ages.

The animals in Groups D and E gained more during the third and fourth years of life than did the animals in Groups A, B, C, or F. At 3 and 4 yr. of age the body weight of animals in Groups D and E had a larger percentage increase over their weight at 2 yr. of age than did the animals in Groups A, B, C, or F (bottom portion, Table 3).

Those in Groups A, B, and D gained the same from 2 to 3 yr. of age when fed Ration 1 as when fed Ration 2, whereas those in Group C on Ration 2 gained somewhat less than those on Ration 1 (98 vs. 112%—bottom portion, Table 3). At 4 yr. of age those that had been fed Ration 2 during the first lactation had gained slightly less than those fed Ration 1.

These results extend and confirm the results of other investigators showing that a restriction of growth during the rearing period results in animals that are slightly smaller at the older ages than heifers that are full-fed (1, 7). The difference observed in body weight at 2 yr. of age progressively decreases with advancing age and, in some instances, may eventually become nil.

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INTAKE AND VALUE FOR MILK PRODUCTION OF OAT SILAGES ENSILED AT THREE STAGES OF MATURITY AND PRESERVED WITH SODIUM METABISULFITE [1, 2]

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SUMMARY

The intake and value for milk production of oats ensiled at three stages of maturity: boot, early-milk, and soft-dough, were compared with each other and with an alfalfa-grass mixed silage. Twelve milking cows were used in an extra-period 4 × 4 Latin square changeover design. In this experiment, the oat silages supplied 86.6–88.0% and the alfalfa-grass silage 86.1% of the total forage dry matter intake. Dry matter intake of the soft-dough stage oats was the highest, but the TDN intake was higher with the boot-stage oats. Differences between oat silages for milk production were correlated with differences in TDN intake from the silages. Acceptable silages were made at all three stages of maturity. It was concluded that the optimum stage to ensile oats is at the boot stage or soon thereafter. Sodium metabisulfite was a satisfactory preservative.

The fundamental objective underlying all attempts to conserve oats as a forage crop is to effectively preserve the nutrients in a palatable condition. However, oat silage feeding to dairy cattle generally has been disappointing. Chemical analyses have indicated marked changes in composition of oat forage with advance in maturity when harvested as hay (9) or silage (11). The optimum stage to harvest the oat plant as a feed, and the proper methods for ensiling to ensure a quality product, have not been established. It has been shown that additives or preservatives are beneficial when ensiling certain green crops (12). Hill *et al.* (3) found that the boot stage was the most favorable stage for obtaining a desirable silage fermentation without the use of an additive. For milk and dough stages, an additive was desirable. McCullough *et al.* (8) obtained optimum preservation and feeding value at the prebloom stage and concluded that ground snap corn was a beneficial preservative but that sodium metabisulfite was unsatisfactory. Thurman *et al.* (11) concluded that oats should be ensiled between the boot and milk stage.

The purpose of this investigation was to compare the intake and value for milk production of oats ensiled at three stages of maturity and preserved with sodium metabisulfite.

EXPERIMENTAL PROCEDURE

Clintland oats were harvested for silage at three stages of maturity: (I) boot, just prior to complete emergence of the inflorescence from the sheath, (II) early-

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milk, and (III) soft-dough. An alfalfa-Ladino-bromegrass mixture (IV) was ensiled as a control silage when the alfalfa was in the 1/10 bloom stage. All forages were direct-cut, treated with 6–8 lb. of sodium metabisulfite per ton, and ensiled in upright steel silos. Sodium metabisulfite has been shown to be an effective preservative (2), and was used because it contributed no food value.

Intake and milk production were studied, using 12 lactating cows in the period between peak-lactation and mid-gestation. The cows were divided into three groups of four cows each, so that animals within a group were producing at comparable levels. A balanced extra-period 4×4 Latin-square changeover design with three squares was used (7). The three groups were allotted to three squares and the animals were randomly assigned to the treatment sequences.

The treatment periods were 15 days in length, composed of a seven-day adjustment period followed by an eight-day experimental period. The final analyses were made on the data from the eight-day experimental period.

Immediately prior to the experiment, the animals were allowed 2 wk. to adjust to the experimental feeds and routine. The silages were fed in ad libitum amounts five or six times daily. A grain mixture containing 18.8% crude protein was fed at the rate of 1 lb. of grain for each 6 lb. of fat-corrected milk (FCM) produced. The composition was as follows, in pounds: ground corn, 400; ground oats, 200; soybean oil meal, 200; steamed bone meal, 8; and iodized salt, 8. Changes in amount of grain fed were made at the beginning of each treatment period, based on production during the preceding experimental period. Good-quality alfalfa hay was fed at 0.3 lb. daily per 100 lb. body weight, based on weights obtained three consecutive days prior to the first treatment period.

During the experiment, the cows were weighed at the same time of day on the last three days of the seven-day adjustment period and the last three days of the eight-day experimental period.

Silage samples were taken weekly for determination of moisture, titratable acidity, and pH. The per cent moisture was determined by the toluene distillation method. The samples were stored in a deep freeze; later, they were composited and oven-dried. Chemical analyses of the silages, hay, and grain were made, using accepted A.O.A.C. procedures (1). The proximate analyses of the grain, hay, and silages are shown in Table 1.

The data were analyzed according to the statistical procedures outlined by Lucas (7). Individual differences among silages in dry matter intake and FCM

TABLE 1
Proximate analyses of grain, hay, and silages

Feed	Dry matter	Crude protein	Crude fiber	Ether extract	Ash	N.F.E.	pH	Titratable acidity
	(%)							
Silage I	21.0	2.33	6.74	1.13	2.08	8.75	4.40	0.79
Silage II	23.5	2.56	7.21	0.92	2.46	10.34	4.65	0.57
Silage III	29.9	2.87	8.97	1.06	2.96	14.04	4.60	0.84
Silage IV	25.5	3.08	9.25	1.07	2.06	10.02	4.39	1.11
Grain mix	90.7	18.78	5.80	3.17	4.90	58.04	.....	.....
Hay	90.7	14.79	33.36	2.17	5.90	33.80	.....	.....

produced were tested for significance with the Neuman-Keuls Sequential Range Test (4).

Total digestible nutrient (TDN) values were obtained for the oat silages, using nine 8-mo.-old Holstein bulls which had been on high roughage programs. Three animals were assigned at random to each of the oat silages. The procedure for a conventional digestion trial was followed, with the exception that plastic bags were used for collecting the feces (10). A 21-day preliminary period was followed by a seven-day fecal collection period.

RESULTS AND DISCUSSION

The oat silages used in the experiment were readily consumed and supplied 86.6-88.0%, and alfalfa grass silage 86.1%, of the dry matter intake from forage. The average daily feed intakes are presented in Table 2. Analyses of variance of silage dry matter intakes (Table 3) show a significant difference between silages. The dry matter intake of Silage III was significantly higher than that of Silages I, II, and IV. The differences in intake among Silages I, II, and IV were small and not significant. Even though the cows consumed 3.5 lb. more dry matter daily when fed Silage III as compared to Silage I, the daily TDN intake was 0.98 lb. greater from Silage I, because the TDN value of the dry matter (10) was 67.1 and 54.1 for Silages I and III, respectively. The TDN intake from the silages ranged between 69.6–74.9% of the total.

TABLE 2
Average daily feed intake, milk production, and body weights

Silage	I Boot	II Early-Milk	III Soft-Dough	IV Alfalfa-grass
Feed consumption				
Silage dry matter *(lb.)*	21.90	21.00	25.40[a]	19.90
TDN from silage *(lb.)*	14.68	12.79	13.70	11.12
Grain *(lb.)*	4.13	3.86	4.00	4.06
Hay *(lb.)*	3.57	3.59	3.84	3.55
FCM produced *(lb.)*	23.30[b]	21.10	22.20	21.70
Lb. TDN/lb FCM	.84	.83	.84	.74
Body weight *(lb.)*	1,190	1,186	1,189	1,182
Weight change *(lb.)*	+.42	+.24	−1.21	−.85

[a] Significantly different from Silage I ($P \leqslant 0.05$) and from Silages II and IV ($P \leqslant 0.01$).
[b] Significantly different from Silage II ($P \leqslant 0.05$).

TABLE 3
Analysis of variance of data on average daily silage dry matter intakes

Source	Degrees of freedom	Mean square	F value
Total	59		
Square	2	442.20	61.10[a]
Cow in squares	9	13.12	2.91
Periods	4	2.31	< 1
Periods × squares	8	3.77	< 1
Direct effect	3	80.00	17.66[a]
Direct effect × square	6	9.86	2.17
Residual	3	11.19	2.47
Residual × square	6	3.96	< 1
Error	17	4.53	

[a] Significant at the 0.01 level of probability.

There is evidence that the type of fermentation will affect the acceptability of the resulting silages. Therefore, dry matter intakes are influenced by fermentation characteristics as well as by the nutritive value of the forage. Laboratory evaluation indicated that the silages had undergone satisfactory fermentations. None of them had offensive odors. They were not slick or slimy to the touch. The color was good, indicating that they had not developed excessively high temperatures. The pH and titratable acid values (Table 1) were representative of silages treated with sodium metabisulfite. The boot-stage silage developed a lower pH than the soft-dough or early-milk stage silages. These results are in agreement with previous findings that oats harvested during the boot stage developed lower pH values (3). In disagreement with the suggestion that sodium metabisulfite is not a satisfactory preservative for oat silage (8), animal acceptance of the oat silages indicated that sodium metabisulfite was an effective preservative for making oat silages at these stages of maturity.

The treatment means for daily FCM are presented in Table 2. The cows on Silage I produced more FCM than those on the other silages. The only significant difference was between Silages I and II ($P \leqslant 0.05$). The FCM for the other two silages was intermediate. The differences between oat silages in milk yield were correlated with differences in TDN intake. The similarity in TDN from the three oat silages is illustrated by TDN consumption per pound of FCM produced (Table 2). The more efficient conversion of TDN from the grass-legume silage may be a reflection of a more favorable nutrient content or combination. Thus, intake, subjective evaluation, and chemical composition are only partly reliable measures for comparing the milk-producing potential of oat silage and other types of silage. The low value of TDN from oat silages for milk production has also been observed by other workers (5, 6).

The extra-period Latin square changeover design permitted the measurement of carryover effect (7). The analyses of variance of silage dry matter intakes (Table 3) and FCM (Table 4) showed that, if present, any carryover effects were essentially completed within the first seven days on a new treatment. Although this design would have permitted the adjustment of the means to eliminate carryover, this was not necessary. The length of time required to

TABLE 4

Analysis of variance of data on average daily fat-corrected milk produced

Source	Degrees of freedom	Mean square	F value
Total	59		
Square	2	1,070.90	285.04[b]
Cow in squares	9	125.72	33.46
Periods	4	95.22	25.34
Periods × square	8	2.72	1
Direct effect	3	12.25	3.27[a]
Direct effect × square	6	2.79	1
Residual	3	4.07	1.08
Residual × square	6	2.00	1
Error	17	3.75	

[a] Significant at the 0.05 level of probability.
[b] Significant at the 0.01 level of probability.

eliminate the carryover effect in a particular experiment would, no doubt, be markedly influenced by the factors being studied.

Although the cows were weighed at regular intervals, the significance of the values obtained (Table 2) is subject to doubt because of the short experimental periods. Also, the values obtained do not differentiate between true and pseudo body weight changes.

The data suggest that acceptable oat silages can be made at all of the stages of maturity studied, and that sodium metabisulfite is a satisfactory preservative for making silages with excellent fermentation characteristics. Also, they indicate from the standpoint of fermentation and milk production that oats should be harvested for silage at approximately the boot stage, or soon thereafter. Since oats often are used as a nurse crop for new seedlings of legumes and grasses, the earlier removal of the oats would conserve soil moisture and give the legume or grass seeding a better opportunity for growth. It would appear that the differences between these oat silages are due essentially to the amount of TDN supplied. The TDN in silage made from Clintland oats was not used as efficiently for milk production as the TDN from the legume-grass silage.

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A COMPARISON OF ZERO, MODERATE, AND LIBERAL LEVELS OF GRAIN FEEDING FOR LACTATING COWS ON PERMANENT, IMPROVED, AND SUPPLEMENTAL PASTURE [1]

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SUMMARY

Two grain-feeding trials, involving the continuous employment of 24 and 18 lactating dairy cows, were conducted during the 1956 and 1957 pasture seasons. Three types of pasture were utilized in each of the two seasons. The feeding of a moderate (5–6 lb.) or a liberal (10–12 lb.) amount of grain had no significant effect on milk production or on body weight.

Grain feeding caused a reduction in the consumption of pasture dry matter of 1.8–3.8 and 3.0–7.5 lb. at the moderate and liberal rates of grain feeding. The net gains in total dry matter consumption were 1.3 and 1.4 lb. for the moderate and liberal grain levels in 1956. In 1957, these values were 2.4 and 5.5 lb., respectively. The different response from grain feeding in the two seasons likely was due to a lower-quality pasture in 1957.

In both trials, there was a highly significant difference in average dry matter digestibility among pastures in favor of orchardgrass–Ladino clover and orchardgrass–Ladino clover plus Sudan grass over Kentucky bluegrass–white clover pasture. Protein digestibility was greater during the 1956 pasture season, probably as a result of the greater and more even distribution of rainfall.

Efforts to minimize the summer slump in milk production largely have been concerned with methods of maintaining a high nutrient intake. Seath and Miller (13) reported a considerable reduction in the grazing time of cattle during hot weather. This finding suggested a need for supplying additional feedstuffs to compensate for what may have been a decreased pasture consumption.

Supplemental feeding of hay or silage to cows on good pasture has been shown by Seath *et al.* (10, 11, 12) and Cole *et al.* (3) to have little effect on milk production or body weight. The ingestion of supplemental feedstuffs apparently was accompanied by a corresponding reduction in forage intake.

Traditionally, grain feeding has been considered as a means of filling the gap between a cow's total energy requirement and the amount of energy the animal can obtain from roughages. Previous work (4, 6, 7, 8) has shown little benefit from grain feeding, but few data are available to indicate the reason for this lack of response. Because of the inadequacy of experimental data relating to the supplementation of summer pastures with varying amounts of grain, this experiment was undertaken to measure the influence of grain feeding on forage dry matter intake and digestibility, protein digestibility, total dry matter intake, milk production, and body weight of grazing, lactating dairy cows.

EXPERIMENTAL PROCEDURE

This experiment was conducted during the 1956 (Trial 1) and the 1957 (Trial 2) pasture seasons. The trials lasted 16 and 20 wk., respectively. Trial 2

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involved the continuous use of 18 lactating dairy cows and the intermittent use of additional dairy cows as put-and-take animals to give the pastures optimum management. These cows were divided into three groups of six animals each. Group 1 was assigned to a permanent pasture consisting of a mixture of Kentucky bluegrass and white clover. Group 2 was assigned to two improved orchardgrass–Ladino clover plots and Group 3 to two similar plots used in conjunction with Sudan grass as a supplemental pasture. Two cows from each group received no grain, two received a moderate amount of grain (6 lb. daily in Trial 1 and 5 lb. daily in Trial 2), and two were fed a liberal amount of grain (12 lb. daily in Trial 1 and 10 lb. daily in Trial 2). Trial 1 differed from Trial 2 in that six additional cows were placed in Group 1, resulting in four instead of two cows on each grain level. The grain ration contained 12% crude protein and consisted of corn and cob meal, wheat bran, ground oats, salt, and steamed bone meal.

Digestion trials were conducted during each 2-wk. interval in Trial 1 and during each 4-wk. interval during Trial 2. Digestibility and dry matter intake were determined by the fecal chromogen–chromic oxide technique (5, 9, 14). In Trial 1, each cow received a gelatin capsule containing 10 g. of chromic oxide orally at 3:30 P.M. daily throughout the experimental period. In Trial 2, 5 g. of the indicator was administered twice daily at 6 A.M. and 3:30 P.M. A five-day collection period was used in both trials. Fecal grab samples were taken at 6 A.M. and 4 P.M. and composited for analyses. Analyses of fecal, forage, and grain samples for protein were made by the boric acid modification of the Kjeldahl method (1). The dried fecal samples were analyzed for chromic oxide by the method of Bolin *et al.* (2).

Daily milk weights were recorded for each animal. Milk samples were taken for four consecutive milkings each week and composited for butterfat determinations every 2 wk. Body weights were obtained three consecutive days, at the beginning of each trial, at the end of each 28-day period, and at the conclusion.

RESULTS

Dry matter intake and digestibility. The dry matter intake from pasture alone is shown in Table 1. The addition of grain to the ration was accompanied

TABLE 1
Effect of grain level on dry matter intake from pasture

Type of pasture	Grain levels					
	1956			1957		
	Zero	Moderate	Liberal	Zero	Moderate	Liberal
	(lb/1,000 lb. body wt.)					
Permanent	31.7	26.1	23.5	28.0	30.0	26.0
Improved	31.6	28.0	25.8	30.5	24.7	30.7
Improved plus supplemental	32.0	31.7	24.4	30.3	28.7	23.0
Average	31.8	28.0	24.3	29.6	27.8	26.6
Average change due to grain feeding		−3.8*	−7.5**		−1.8	−3.0

* Significant ($P < .05$).
** Significant ($P < .01$).

by a decreased consumption of pasture forage. In the 1956 trial (Trial 1), the reduction in pasture dry matter intake was significant ($P < .05$) for the moderate grain level and highly significant ($P < .01$) at the liberal grain level. The same trend was observed in Trial 2, but the differences were not statistically significant.

The total dry matter intakes among grain levels for both trials were not significantly different (Table 2). The dry matter intake from grain in Trial 1 was 5.1 and 8.9 lb/1,000 lb. body weight for the moderate and liberal grain levels, respectively. In the same order, the average for Trial 2 were 4.2 and 8.3 lb. The net gains in total dry matter intake were 1.3 and 1.4 lb. for Trial 1 and 2.4 and 5.3 for Trial 2. These increases in dry matter intake are considerably smaller than the weight of the dry matter furnished by the grain.

The feeding of grain had no apparent effect on the dry matter digestibility of the forage (Table 3). Forage dry matter digestibility was calculated by assuming a constant digestibility (71%) for the grain and correcting the fecal chromogen value accordingly. During both trials there was a highly significant ($P < .01$) difference in dry matter digestibility in favor of the two improved pastures over the permanent bluegrass-Ladino pasture. However, the magnitude of this difference was quite small.

TABLE 2
Effect of grain level on total dry matter intake

Type of pasture	Grain levels					
	1956			1957		
	Zero	Moderate	Liberal	Zero	Moderate	Liberal
	(lb/1,000 lb. body wt.)					
Permanent	31.7	31.2	32.5	28.0	33.9	35.2
Improved	31.6	33.2	34.2	30.5	29.0	39.1
Improved plus supplemental	32.0	36.6	33.8	30.3	33.0	30.6
Average	31.8	33.1	33.2	29.6	32.0	34.9
Average change due to grain feeding		+1.3	+1.4		+2.4	+5.3

No significant differences.

TABLE 3
Effect of grain level on forage dry matter digestibility

Type of pasture	Grain levels						
	1956			1957			
	Zero	Moderate	Liberal	Zero	Moderate	Liberal	Average
	(%)						
Permanent	67.2	67.2	66.3	63.4	65.2	63.6	65.5
Improved	71.8	71.2	72.1	67.5	67.0	68.6	69.7**
Improved plus supplemental	70.7	70.7	69.8	65.6	65.4	66.9	68.2*
Average	69.2	69.0	68.6	65.5	65.9	66.4	67.4

* Significant ($P < .01$ in 1956) ($P < .05$ in 1957).
** Significant ($P < .01$).

Milk production. During both trials, the feeding of grain apparently resulted in a slight increase in milk production (Table 4). When tested by the analysis of covariance, to remove the influence of level of initial production, these differences were not significantly different. The average 4-wk. persistency for the zero, moderate, and liberal levels of grain feeding, respectively, were 90.8, 89.1, and 92.9% for Trial 1, and 84.4, 88.0, and 91.2% for Trial 2. No statistical significance was found among these values. A highly significant ($P < .01$) difference in persistency was obtained during the third 28-day period in Trial 2, when grain feeding exerted a beneficial effect. During this period the pastures became short and of low quality. Kind of pasture apparently had little effect on milk production during either trial.

Body weight. There was considerable variation in body weight changes among all the animals, regardless of treatment. The data in Table 5 represent

TABLE 4

Average milk production for week prior to experiment and for entire experiment

Type of pasture	Grain level	Average daily 4% FCM			
		1956		1957	
		Preliminary	16-wk. experiment	Preliminary	20-wk. experiment
		(lb.)			
Permanent	Zero	43.1	29.8	40.7	27.6
	Moderate	35.2	26.1	50.0	36.1
	Liberal	41.6	33.2	38.3	31.8
Average		39.9	29.7	43.0	31.8
Improved	Zero	44.0	41.1	48.6	32.4
	Moderate	33.5	34.2	32.0	26.0
	Liberal	42.2	46.3	48.8	43.1
Average		39.9	40.5	43.1	33.8
Improved plus supplemental	Zero	35.6	32.2	40.8	26.4
	Moderate	53.7	44.3	48.4	38.6
	Liberal	36.5	31.8	44.1	33.1
Average		41.9	36.2	44.4	32.7
Average of all pastures	Zero	41.4	33.2	43.4	28.8
	Moderate	38.4	32.7	43.5	33.6
	Liberal	40.5	36.1	43.7	36.0

No significant differences.

TABLE 5

Effect of grain level on body weight changes during entire experiment

Type of pasture	Grain levels					
	1956			1957		
	Zero	Moderate	Liberal	Zero	Moderate	Liberal
	(lb/cow)					
Permanent	+ 2	+68	+ 57	− 20	+ 44	+ 19
Improved	+18	+82	+ 83	+ 28	+124	+ 90
Improved plus supplemental	+33	− 4	+112	+122	+ 47	+136
Average	+14	+53	+ 77	+ 43	+ 71	+ 82

No significant differences.

the average differences between the body weights at the beginning and at the completion of the trials. During both trials the grain-fed groups tended to gain more weight than the controls. However, this trend was not consistent enough to be statistically significant. It is of interest to note that all the groups tended to gain weight during the trials.

Protein digestibility. There was no significant difference in protein digestibility of the total ration associated with level of grain feeding. Trial 1 averages were 70.7, 71.5, and 70.5% for the zero, medium, and liberal grain-fed groups, respectively. In the same order for Trial 2, the values were 65.5, 65.9, and 66.4%. The lower values for Trial 2 were attributed ot a lack of sufficient rainfall to maintain succulent herbage.

DISCUSSION

Under the conditions of this experiment, the feeding of grain to lactating dairy cows on good pasture failed to cause a significant increase in milk production or body weight. In both trials, however, the cows fed a high level of grain produced more milk and gained more weight than the cows on the zero grain level. In Trial 1, the cows receiving the medium grain level produced less milk than the controls, but gained slightly more in body weight. In Trial 2, the cows in this group excelled the controls both in milk production and in body weight. Grain feeding apparently was more effective in Trial 2 than in Trial 1, since grain feeding in Trial 1 caused a significant decrease in pasture consumption but did not do so in Trial 2. The results of the two trials may differ because of differences in pasture quality. This difference may have been due to the higher and more evenly distributed rainfall in 1956.

The rainfall in 1956, from March through September, was 33.2 in. as compared with 26.7 in. during the same period in 1957. The 50-yr. average for this area is 26.3 in. during these months. The rainfall for July, 1957, however, was only 0.76 in. as compared with a 50-yr. average of 4.3 in., and 6.1 in. in 1956. The grain-fed cows produced significantly more milk than the controls during July, 1957. During this period the pastures were of very poor quality.

The apparent lack of response to grain feeding may be due to the reduction in consumption of pasture herbage, especially during periods of abundant rainfall and lush pastures. These results are in agreement with Cole *et al.* (3), who reported a 13% reduction in pasture dry matter intake among cows receiving alfalfa hay as a supplement. Seath *et al.* (12) have reported a 32% decrease in pasture consumption to result from supplemental feeding of hay.

These results indicate there is little basis for the belief that supplemental feeding of grain during the good pasture season will significantly help maintain milk production. It must be considered, however, that the pastures used in this experiment were of very high quality and that the grain-fed cows tended to produce slightly more milk and to gain more weight. Economic consideration of the data indicated that grain feeding was not profitable, especially during Trial 1. It remains to be determined experimentally just how low pasture quality must become before grain feeding can be considered a profitable practice.

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ESTROGENIC ACTIVITY IN MILK OF COWS AND THE BILE OF CALVES FED LOW LEVELS OF DIETHYLSTILBESTROL[1]

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SUMMARY

Milk from cows fed diethylstilbestrol (DES), and bile from calves fed this substance, were extracted and tested in castrate mice, using uterine weight as the criterion for measuring estrogenic activity.

The following conclusions were drawn:

Extracts of milk from untreated cows, in different phases of sexual cycles, gave no estrogenic response.

Extracts of milk from cows fed 10 mg. diethylstilbestrol (DES) per 1,000 lb. body weight per day caused no measurable estrogenic response.

Test mice consistently responded to known amounts of 0.048 mg. DES each.

Known amounts of DES were placed in milk, then extracted. These extracts caused consistent responses for estrogenic substances in test mice.

Bile from calves that had been given 10 mg. DES daily was extracted. This extract in test mice caused responses indicating that a daily collection of bile contained 46.08 μg. of free DES or its equivalent of estrogenic substance. Bile extracts from an untreated male calf indicated no estrogenic response.

While studying the influence of diethylstilbestrol (DES) in dairy cows (1) it became important to know if it were excreted in the milk. Determination of DES in milk has received little attention, but Turner (8) states that, ". . . from 10 to 20 mg. per day to cows weighing 100 lb. is not sufficient to permit the passage of significant amounts of estrogen into the milk." Voelker and Becker (9) detected little or no estrogen in the milk of cows that had received implants of 15 and 30 mg. of diethylstilbestrol, but found considerable amounts of estrogen in the milk of cows implanted with pellets of 240 and 1,500 mg. of DES. The analysis of muscle tissue of animals fed DES indicates that little if any of this material is deposited in the tissues when given in moderate dosage (2, 4-7).

EXPERIMENTAL PROCEDURE AND RESULTS

Preliminary testing for estrogen was begun, using milk from 17 cows that had been fed from 0 to 15 mg. DES per 1,000 lb. body weight per day. They varied in age, and stages of lactation and pregnancy. In all cases in which milk was extracted in this study, one each of five pairs of identical twin cows was fed 10 mg. DES per 1,000 lb. body weight per day, beginning on the 56th day of lactation. The opposite member served as control. Milk was tested for estrogenic material after the cows had received the DES for 2 mo. or longer.

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Detection of DES was attempted, using mice as the assay animals. In each test, uterine weight was taken as the basis for evaluation.

Female mice were castrated within a few days after 6 wk. of age and used when approximately 8 wk. old, in testing for estrogens. First trials were conducted by feeding the mice dry, powdered milk for 14 days. Milk from untreated cows was fed to 14 mice, and milk from cows that had received 10 mg. DES per day was given to 13 mice. Each mouse consumed approximately 26 g. of the dry milk. Twelve mice were given maintenance feed (Laboratory Chow). Uterine weights were not significantly different among mice fed maintenance feed, milk from untreated cows, or milk from DES-treated cows ($P < 0.001$).

To test for estrogens from a larger quantity of milk per mouse, the milk was extracted. Extraction of the milk was done by refluxing with methanol, as described by Pope and Roy (3). However this procedure extracts only the free estrogens. The dried methanol extract was dissolved in 12 cc. of olive oil and injected subcutaneously into castrate mice, 0.1 cc. daily for four days. The mice were killed 48 hr. after the last injection and their uteri removed. Each uterus was severed as closely as possible to the cervix, trimmed of any fat, and blotted with filter paper. Weighing was done within 1 to 2 min. after removal.

Extracts from 13.33 g. of milk and also 14, 25, 28.5, 47.5, and 80 g. of milk from DES-treated cows were given to each mouse, with no significant increase in uterine weight. These uterine weights were compared with those from mice treated with extracts of milk from untreated cows and mice that were uninjected. With each series of mice tested with milk extracts, other mice were injected with known amounts of DES in doses of 3.12, 0.388, and 0.048 μg. Dosage was regulated to give a recognizable increase in uterine weight but not more than maximal stimulation. Typical responses in groups of mice were uterine weights of 62.90, 45.32, and 22.61 mg., respectively. Untreated mice and those injected with milk extracts had uterine weights averaging 13.18 mg. In almost all tests, seven mice were treated alike, to constitute a group. More than 130 mice were used in specific tests, in addition to others used in numerous preliminary trials.

To test the process of milk extraction, 21.84 μg. of DES was added to 100 g. of milk and extraction conducted as usual. Extract from the equivalent of 13.33 g. of milk per mouse resulted in an average uterine weight of 31.97 mg. Extract from this same sample of milk without the DES added gave uterine weights of 13.44 mg. Extracts from 80 g. of milk (cows fed 10 mg. DES per day) were given to a single mouse, but in no case was there increased uterine weight in the test mice. Mild responses (uterine weights averaged 19.30 mg.) were recognized by giving extracts of milk from cows fed 20 mg. of DES per day.

Extracted milk, from cows given 10 mg. of DES per 1,000 lb. of body weight per day, showed no response in test mice, so the search was then directed to the bile.

Four male calves were used in this phase of the study; each weighed approximately 180 lb. Each calf was given orally 10 mg. DES per day, beginning four days before collections of bile were made and continuing on through the collection period of four to six days.

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To make collections, a bile fistula was prepared. The fistula was made by first anesthetizing the calf with Nembutal, given intravenously. An incision was made just back of the last rib and parallel to it on the right side. The bile duct was ligated and the bile in the gall bladder was withdrawn with a hypodermic needle and syringe. A small incision was made in the end of the gall bladder and an 18-gauge hypodermic needle inserted. The needle had been prepared by soldering a knob on it near the end, to prevent its slipping out. The gall bladder was then closed by tying it around the needle back of the knob. A sterile rubber tube led from the needle out through the body wall and was attached to a rubber basketball bladder that served as a collecting bag. A cloth band around the abdomen of the calf supported the weight of the bag and its contents.

Twenty-four-hour collections were made. The bile was extracted by members of the University Department of Chemistry, using a liquid extractor in which diethylether was used as the solvent. This method extracts only the free estrogens, leaving any protein-bound and other conjugated estrogens with the remainder of the bile. The dried ether extract was dissolved in olive oil for injection into the test animals.

The bile extract was tested, following the procedure for the milk extracts. The test mice were injected subcutaneously with 0.1 cc. of the extract daily for four days. They were killed 48 hr. after the last injection and the uteri promptly removed and weighed.

The extract from a daily collection of bile was dissolved in 12 cc. of olive oil. To bring the quantity of estrogenic material into a range that could be tested, dilutions ranging from 1:32 to 1:128 were tried. The dilution of 1:128 produced what was considered a minimal response, since uterine weights in the tested mice were slightly greater (statistically significant) than the untreated ones. A dilution of 1:32 resulted in uterine weights nearest to those from the known amount of DES of 0.048 μg., so it was used for final evaluation. Each test mouse was given 0.4 cc. of the oil preparation. From these values it was concluded that a daily collection of bile contained 46.08 μg. of DES (or equivalent of estrogenic material).

The 46.08 μg. of DES cause considerable estrogenic activity in terms of mouse uterine response, but far short of the 10 mg. daily dosage of DES given the calf. The estrogenic response was not determined to be from DES, as such. The fate of the remaining estrogen is not known, since fecal or bound estrogen determinations were not made. This study reveals, however, that estrogenic material appears in the bile of calves that have been fed DES. It further gives an estimate of the amount of free estrogen passed on in the bile.

Bile from one male calf that had not received DES was extracted and tested, but no estrogenic activity was found.

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THE PHYSIOLOGICAL BEHAVIOR OF IRON IN THE CALF

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SUMMARY

Results of ferrokinetics studies and tissue distribution investigations as a function of time after Fe-59 administration in 21 calves indicate radiochemical procedures to be a sensitive measure of integrity of erythropoietic tissue. They further demonstrate feasible measurements of physiological mechanisms of iron metabolism that should provide means for dietary requirement estimations and basic data essential for understanding the nature and pathogenesis of prevalent anemias among farm animals.

The importance of iron in the nutrition of man and laboratory animals has led to numerous investigations concerned either directly or indirectly with the metabolism of this element (6, 7, 11). However, relatively few reports in the literature (1, 3, 4, 8) have dealt directly with the physiological behavior of iron in the ruminant. Availability of radioiron and development of scintillation counting methods have made possible a sensitive measurement unattainable by classic methods for the investigation of ferrokinetics (6, 10). Calves were employed because of the need for additional information on blood formation in the ruminant and because of the economic importance of the species. The objectives of this investigation were to determine the movement of labeled iron in the normal calf as a function of time and to interpret such data in terms of behavior and utilization of stable iron by the erythropoietic tissue.

METHODS AND MATERIALS

Twenty-one selected dairy calves with similar nutritional background, ranging in age from 3 to 7 mo., were maintained on a full feed of grass hay and commercial dairy feed throughout the experimental period. Radioisotope procedures and management of the animals previously have been discussed by Hansard and Foote (4). However, in brief, following the preparation period, each animal was oriented in a metabolism unit and a blood sample drawn from the jugular vein, centrifuged, and the plasma incubated 30 to 60 min. with 10 to 40 μc. of high specific activity Fe-59 citrate. A known volume of the homologous labeled plasma was quantitatively reinjected into the immobilized animal and, after mixing had occurred, periodic blood samples were taken and radioactivity of whole blood and plasma measured in 4-ml. aliquots for calculation of blood volume, plasma iron clearance, and for the ferrokinetic data (1, 6, 7). Total plasma iron was measured by the method of Barkan and Walker (2) and hemoglobin was determined routinely by standard procedures. Following designated intervals of time, the animals were killed and selected tissue samples taken im-

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mediately for radioassay and for total iron determinations by Wong's (12) procedure. For convenience of presentation and for comparative purposes, Fe-59 data from all animals were calculated and standardized on the basis of a 100-lb. calf.

RESULTS AND DISCUSSION

A summary of the blood values for the calves in this study is shown in Table 1. These averages for hemoglobin, plasma iron, total blood, red cell, and plasma volume compare favorably with values in the literature. However, the range was wide and indicates the need for studies with increased numbers for establishment of firm values for this species. This was especially evident for plasma iron values, a key value for ferrokinetic calculations, and strongly suggests a diurnal effect in the calf.

TABLE 1
Summary of blood values for experimental calves

	Mean ± standard deviation
No. of calves	21.0
Av. body weight *(kg.)*	92.6
Total blood volume *(%)*	6.1 ± .4
RBC volume *(ml/kg)*	23.8 ± 3
Plasma volume *(ml/kg)*	36.4 ± 4
Packed RBC volume *(%)*	39.5 ± 3
Hb. *(Gr/100 ml.)*	10.5 ± 3
Plasma iron *(μg/100 ml.)*	240 ± 55

Plasma iron clearance and turnover rate. The plasma serves as a central metabolic pool through which all iron must pass in the process of absorption from the tract, soft tissue and bone, transfer from one tissue to another and for excretion. The kinetics of Fe-59 ion movement in the blood thus has been established as an important index to iron utilization in the animal body (6, 10, 11). Average blood Fe-59 values following injection are shown in Figure 1. The inserted line graph demonstrates a semilogarithmic plot of activity in serial plasma samples and represents removal of radioiron from total plasma of these calves as a function of time after dosing. It was of interest that, with the exception of three calves, all animals demonstrated a two-component plasma iron clearance curve. The first component of this curve has been postulated by Huff *et al.* (6) and Weinstein and Beutler (11) to represent liver iron uptake, and the second component that of bone marrow (7). Explanations for these differences in clearance rate among calves will require further investigation, and will be discussed later in this report. In all cases the second component of the curve was employed in this study, extrapolated to zero using a line derived by method of least-squares. The intercept of this line with the ordinates was used to give plasma activity. The slope of the line was employed to calculate total plasma and blood volume and to determine time in hours at which one-half of the injected dose was cleared ($T\frac{1}{2}$) from the plasma. Mean value of 2.7 ± 0.52 hr. clearance time for the 21 calves was somewhat slower than the 1.55 hr. reported by California workers (1), but may have been due to differences in age or to dietary iron histories.

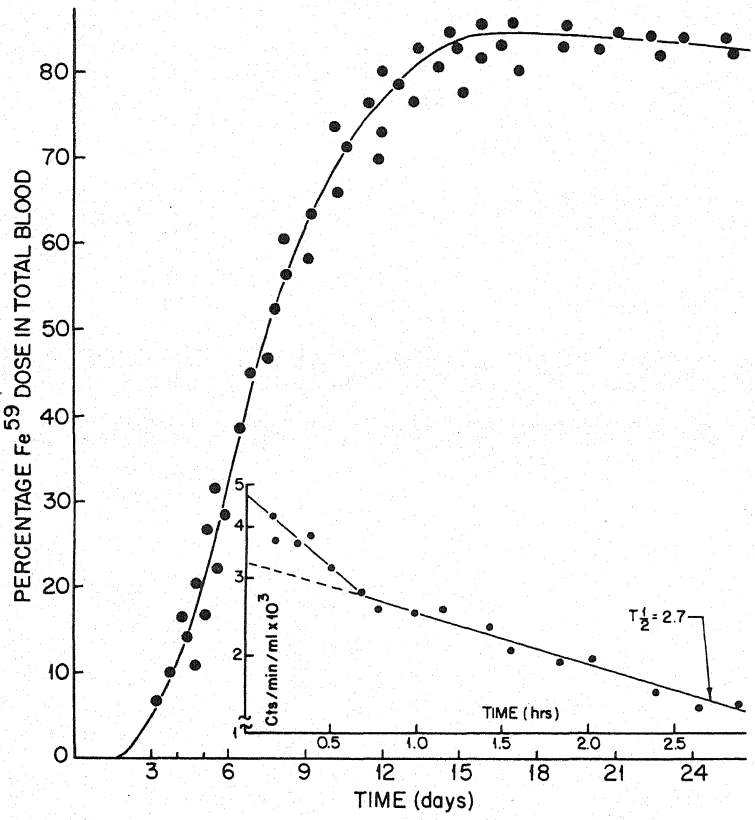
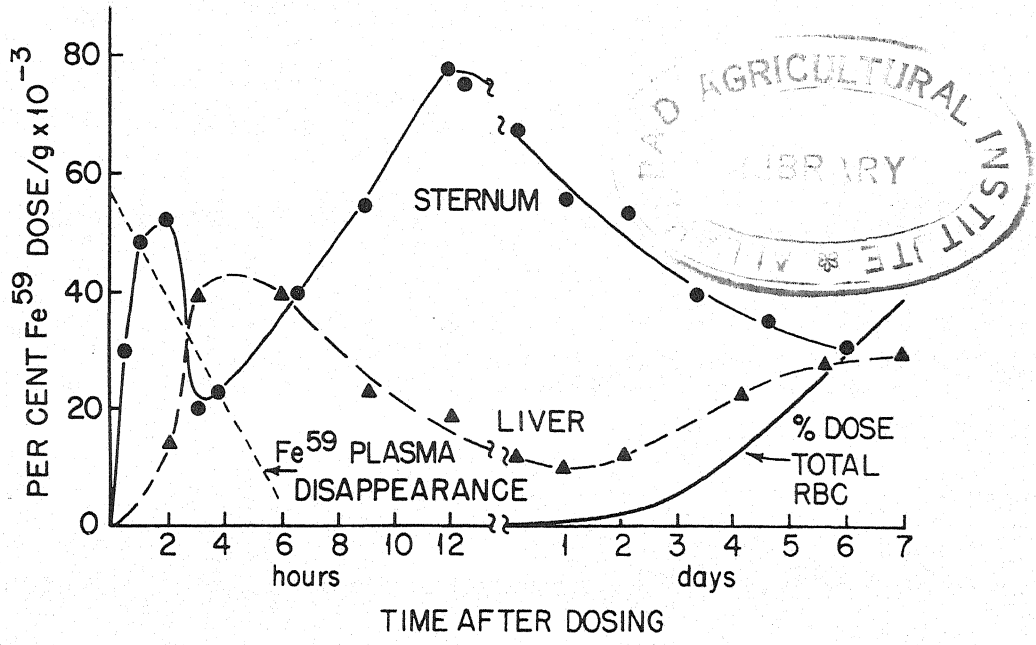


FIG. 1. Removal of radioiron from plasma (insert) and utilization by red cells as a function of time after administration to calves. (Note the dual component curve for plasma iron clearance.)

The slope of this line and the plasma iron concentration values were used to calculate plasma iron turnover rate (PITR) by the method of Huff *et al.* (6). These values averaged 0.54 ± 0.11 mg/kg/day, which were somewhat lower than those reported for swine by Jensen *et al.* (7) and the 0.81 mg/kg reported for cattle by Baker and Douglas (1). However, this difference may be accredited to the observed higher plasma iron and the slower plasma iron clearance values evidenced in the current study.

Red blood cell iron, utilization and turnover. Hemoglobin, venous hematocrits, total plasma iron, and total blood volume were measured routinely, both initially and on the day of peak Fe-59 uptake by the red cells, to minimize obvious errors due to any physiological change in the peripheral blood. These values (Table 1), for the day of maximum $Fe^{59/M}$ utilization, were employed in calculations of the ferrokinetic data reported in Table 2. Hemoglobin levels and total blood volume (TBV) in milliliters were used to calculate total red blood cell iron (RBCI) as follows:

$$\text{RBCI (mg/kg)} = \text{Hb}\left(\frac{\text{TBV}}{100}\right)\left(\frac{3.4}{\text{wt. kg.}}\right).$$



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FIG. 2. Iron concentration in liver and sternum of calves as a function of time after iron dosing. Plasma disappearance and red blood cell utilization curves are superimposed for clarity.

TABLE 2

Summary of calculated ferrokinetic values for experimental calves[a]

Plasma, T½ *(hrs.)*	2.7 ± .52[b]
PITR *(mg/kg)*	.54 ± .11
Iron[59] utilization *(%)*	84.3 ± 15
RBCI *(mg/kg)*	21.4 ± 4.1
RBCIIR *(mg/kg/day)*	.46 ± .08
RBC life span *(days)*	47 ± 8

[a] See text for calculations.
[b] Mean ± standard deviation.

The per cent of Fe-59 dose incorporated into total red blood cells as a function of time after intravenous administration also is shown in Figure 1. Calculations were made daily for the first 2 wk., then biweekly throughout the experimental period by conventional radiochemical procedures described by Hansard and Foote (3). Following a time lag of from 48 to 72 hr. after Fe-59 disappearance from the plasma, it reappeared in the red blood cells, reaching peak concentration of 84.3 ± 15% 12 to 16 days after dosing. Iron utilization at the peak ($Fe^{59/M}$) period was calculated for individual animals from total blood volume (TBV) in milliliters, retained radioactivity (c.p.m/ml), and total dose administered (c.p.m. injected), thus:

$$\text{Iron utilization } (Fe^{59/M}) = 100\left(\frac{\text{TBV} \times \text{c.p.m/ml}}{\text{dose}}\right).$$

Rate of iron incorporation into the red cells each day was presumed to represent rate of erythropoieses, inasmuch as Fe-59 does not enter the mature red cells

directly and is expressed by the RBC incorporation values shown in Table 2. If it is assumed that these animals were in a steady state of red blood cell production and, since the plasma iron turnover rate (PITR), blood volume, and hemoglobin values were calculated from data procured on the day of peak Fe-59 utilization, incorporation rate would be essentially equal to turnover rate (1). The approximation of red blood cell iron incorporation rate (RBCIIR) was obtained, therefore, by multiplying plasma iron turnover in milligrams by percentage of Fe-59 ultimately appearing in the total red cells as follows:

$$\text{RBCIIR} = \text{PITR}\left(\frac{\text{Fe}^{59/\text{M}}}{100}\right)$$

Mean values are given in Table 2. Red cell iron incorporation or turnover amounted to 0.46 mg/kg or 42.6 mg/per day for a 100-lb. calf.

Red blood cell life span. The red blood cell life span (RBCLS) or survival time was estimated by the following formula:

$$\text{RBCLS (days)} = \frac{\text{Total RBC iron}}{\text{RBCIIR}}$$

Estimated red blood cell survival time for the 21 calves averaged 47 ± 8 days, which approximates that reported by other investigators cited and that by other procedures (5) for calculation. However, this value is considerably less than the 115 days reported for calves by another method (9).

Movement of Fe-59 in the body. To determine location and rate of iron in the body of the calf, animals were sacrificed at periodic intervals of 1 hr. to 20 days after Fe-59 administration, for tissue distribution studies. Animals were bled out from the jugular vein and some 20 selected tissues and organs were weighed and sampled in duplicate for radioactivity measurement and total iron determinations. Biopsies from the liver and sternum were taken from several animals to further supplement data at the earlier periods after dosing. Fe-59 concentration and specific activity values of selected calf tissues as a function of time after dosing are shown in Table 3. These data show Fe-59 concentration to be higher in trabecular bone than in soft tissue at all periods of study. Liver re-

TABLE 3

Iron[59] concentration** and specific activity* of selected calf tissues as a function of time after intravenous administration

Tissue	Hours after dose administration					
	1	6	12	24	48	168
Muscle	4(2.8)*	4(2.1)	3(2.4)	4(2.8)	4(2.8)	3(2.4)
Liver	94(1.7)	398(6.2)	205(3.2)	100(1.5)	108(1.6)	110(1.6)
Adrenals	86(13.4)	216(33.6)	91(14.2)	58(9.0)	68(10.6)	59(9.2)
Kidney	46(1.9)	90(15.5)	70(12.0)	40(7.1)	25(4.5)	25(4.5)
Spleen	60(1.4)	170(4.1)	60(1.4)	23(0.6)	28(0.7)	18(0.5)
Small intestines	19(8)	24(10)	57(25)	38(18)	30(13)	20(8)
Sternum	485(35)	382(29)	770(55)	550(40)	450(35)	260(18)
Vertebra (L)	530(46)	277(24)	631(55)	525(46)	692(60)	270(24)

** Fe^{59} concentration expressed as per cent dose $\times 10^{-4}$/g, and * specific activity as concentration of Fe^{59} per gram of total iron $\times 10^{-2}$.

tained the highest concentration of iron among the soft tissue, with adrenals, kidneys, and spleen following in that order. Values for thyroids, pancreas, and muscle were intermediate to low in concentration. It was of interest that concentration in bone was consistently higher than in soft tissue and, except for liver, concentration in trabecular bone was ten to 20 times that in other tissues. After 24 hr., bone appeared to serve as the principal metabolic pool for iron storage. Concentration of Fe-59 per gram of total iron (SA) in all tissue more or less paralleled concentration values, after equilibrium had been reached. However, turnover rates were highest for bone for all periods, with little differences noted between trabecular bone of sternum, rib, vertebra, or pelvis. These values, after 24 hr., were ten to 20 times those of muscle, liver, or spleen. The higher relative values for kidney are not clear, inasmuch as total excretion values indicated less than 1% of the dose to be cleared through this organ. The consistently high iron values for the adrenal were of interest and are being further investigated.

When consideration was given to Fe-59 concentration as a function of time after dose administration, there was notably wide variation among tissues. This difference in Fe-59 concentration between liver and sternum is graphically illustrated in Figure 3. Plasma iron clearance and RBC iron utilization curves are superimposed for reference and clarity. It was of special interest to note that liver Fe-59 increased comparatively slowly, reaching peak concentration 4 to 6 hr. after dose administration; then receded gradually to one-fourth peak levels

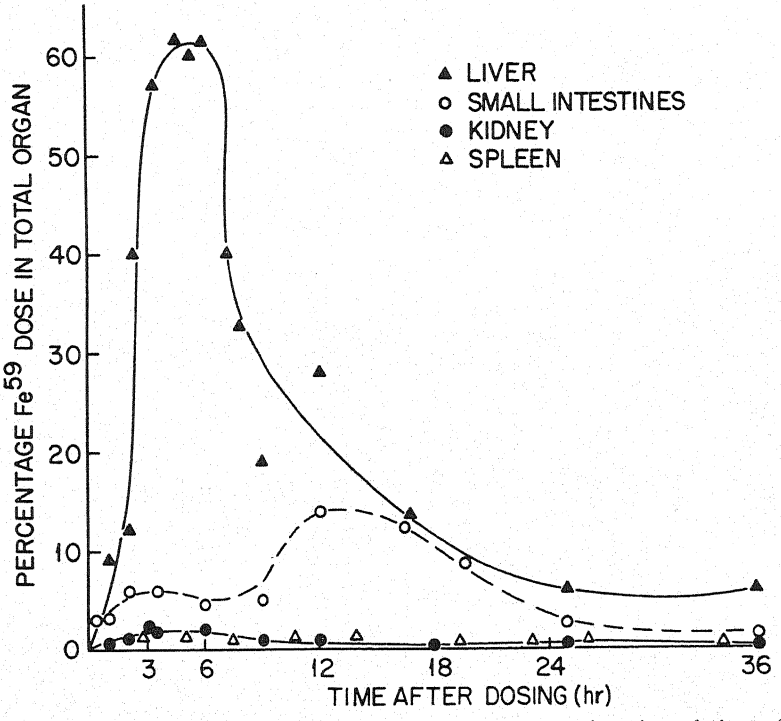


FIG. 3. Radioiron concentration in total organs of calves as a function of time after dose administration.

24 to 48 hr. later. However, concentration in trabecular bone (sternum) increased more rapidly, reaching an initial peak within 1 to 2 hr., receded to less than one-half peak level, before again rising after 12 hr. to four times this level. This phenomenon is yet unexplained, but does indicate (a) bone marrow to have the highest developed physical properties for concentrating iron, (b) that initial rapid plasma iron disappearance is related to bone marrow concentration more than to liver uptake, and (c) that ultimately the initial marrow and liver iron returns to the bone for incorporation and utilization by the red blood cells.

Concentration of Fe-59 in selected total organs of calves is shown (Figure 3), as a function of time after dose administration. The liver contained the most radioactivity, averaging 61% of the administered dose after 5 hr., and decreased rapidly to 6% at 24 hr. Peak levels of 2, 2, and 14% Fe-59 for total kidneys, spleen, and for total small intestines, respectively, occurred at 3, 4, and 12 hr. after a single dose, and indicate the relative and active part played by these organs in the metabolism of iron in the calf.

ACKNOWLEDGMENT

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STUDIES ON THE RELATIONSHIPS BETWEEN RUMEN ACIDS AND FAT METABOLISM OF RUMINANTS FED ON RESTRICTED ROUGHAGE DIETS [1]

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SUMMARY

The feeding of restricted roughage with high levels of concentrates produced significant declines in milk fat in lactating cows and goats. Associated with the decline in milk fat were a signficant increase in the concentration of rumen propionic acid and a decrease in blood ketone bodies. There was a decrease in arterial-blood acetic acid and in the arterial-mammary difference under conditions of ground and restricted-roughage feeding. The feeding of sodium acetate tended to increase low milk fat, and the feeding of sodium propionate tended to lower it further. An hypothesis regarding a mechanism of milk-fat depression based on the antiketogenic properties of propionic acid is discussed.

Since the discovery of Powell (16) of the effect of finely ground or restricted-roughage rations on the milk fat of dairy cows, studies (1, 2, 25, 26) have shown that a proper understanding of this phenomenon bears directly on the fundamental problems of the nature of ruminant digestion and metabolism. These studies may be divided into two categories: first, those on changes in the rumen fermentation and other factors affecting digestion; and second, those on the subsequent metabolism of products from fermentation and digestion. This paper deals with the relationships between ketone body, acetate and propionate metabolism under conditions of restricted roughage feeding.

The digestibility of crude fiber has been shown to be diminished in finely ground roughages (3, 8). Rodrigue (18) found that the rate of passage was increased by very fine grinding. Maki (10) showed a decrease in number of rumen microorganisms of the types that are cellulolytic when cows were fed a high-concentrate restricted roughage diet. Over-all rumen microorganisms increased, however.

Tyznik (26) and Balch (1) have reported a decrease in the proportion of acetic acid in the rumen when these diets are used. It has been hypothesized that low milk fat is due to the fact that the amount of acetic acid produced by fermentation in the rumen is insufficient for synthesis of milk fat by the mammary gland. This hypothesis is based in part on the observation that there is a recovery in milk fat when sodium acetate is fed to milk fat–depressed cows

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(26), and on extensive work (15) concerning the utilization of acetate by ruminant mammary tissue. However, the idea has been contested by McClymont (12), who has determined arteriovenous differences in blood acetic acid and was unable to find expected differences under conditions of low milk fat.

Decreases in the Reichert-Meissl number of the buterfat of cows with low milk fat have been reported (26). By means of arteriovenous differences, Shaw (21,23) has demonstrated that while the active mammary gland uses *beta*-hydroxybutyric acid (BHBA), the nonlactating gland does not use significant amounts of BHBA. The daily production of butter fat has been associated with blood ketone levels (11). Shaw (22) has reported in perfusion studies on isolated bovine mammary gland—using labeled acetate and BHBA—that BHBA is utilized 2.5 times more efficiently than acetate for milk-fat synthesis. Popjak (15) has suggested that 60% of the short chains in butterfat may come from BHBA. In view of the above evidence, it was considered worth while to examine the metabolism of cows which were fed ground or restricted roughage.

EXPERIMENTAL METHODS

Rumen samples were taken by stomach tube. Samples were acidified with 50% H_2SO_4 to a low pH and were filtered through cheese cloth. Then the filtrates were frozen and stored until analyses could be made. Rumen acetic, propionic, and butyric acids were determined by a modification of the chromatographic method of Neish (13). Blood samples were taken either with sodium fluoride or with heparin as the anticoagulant. Protein-free filtrates (Folin-Wu) were prepared and stored in a refrigerator until they could be analyzed. Glucose and ketone body determinations were performed, respectively, by the methods of Benedict (5) and Behre (4). Babcock analyses for milk fat were made on one- or two-day composite samples throughout the studies. Blood acetic acid was determined via the same chromatographic technique used for the rumen samples. The blood filtrates were prepared for chromatography by evaporating between 50 and 100 ml. of neutralized solution to dryness, dissolving the residue in dilute H_2SO_4, and transferring an aliquot to a column. An attempt was made to estimate the amount of such other fatty acids as butyric and propionic. However, the latter acids were present in such small quantities that accurate measurement was not possible. Apparently, most blood propionic and butyric acids absorbed through the rumen wall are quickly removed by the liver (17).

Experiment 1. During a 3-wk. preliminary control period, four cows and six goats, all lactating, were fed all of the long hay they would clean up, plus concentrates according to milk production. Blood samples and rumen samples were taken five times at two- or three-day intervals during the control period. After the animals were changed to the experimental rations, blood and rumen samples were taken at approximately weekly intervals.

The four cows were given restricted roughages (3 lb. hay per day) and concentrates ad libitum. The consumption of concentrates ranged from 20 to 35 lb. per day among the cows. Four goats were placed on restricted roughage and high concentrates, receiving only a small handful of hay a day. Two additional

goats were fed on alfalfa meal ad libitum and were given a normal complement of concentrates.

The data for milk fat, blood glucose, ketones, and rumen-acetic, propionic, and butyric acids have been averaged for the groups of cows and goats and presented (Table 1). Data were analyzed by the "t" test. All the cows and three of the goats showed a definite depression in milk fat when fed on restricted roughage. One goat ceased lactating before any of the effects of the restricted roughage could be noticed. Alfalfa meal as the sole roughage did not appear to depress milk fat when fed to two goats, although milk fat appeared to increase when these two animals were placed back on normal roughage.

Blood ketones showed a significant decrease with milk-fat depression in both cows and goats. Rumen acetic acid did not decrease significantly; however, rumen propionic acid significantly increased when cows or goats were fed the ground or restricted-roughage diets. Rumen butyric acid showed a tendency to decline slightly. This tendency was significant in the cow data.

A series of correlations was calculated according to the method of Snedecor (24) and presented in Table 2. Correlations between various constituents are presented separately for cows and goats, and are divided into two groups—those from animals on normal diet and those from animals on restricted roughage. The degree of milk-fat depression is generally related to the concentration of rumen propionic acid, the development of milk-fat depression being associated with a concurrent rise in rumen propionic acid. This relationship is to be found in the highly significant negative correlations between rumen propionic acid and milk fat obtained from the data on fat-depressed cows and goats. The case of the two goats fed alfalfa meal shows that the converse—that with every rise in rumen propionic acid there is a decline in milk fat—is not necessarily true. However, this may be resolved, as will be shown in the discussion, if the proportion of acetic to propionic acid is considered; it may be seen in Table 1 that rumen acetic and butyric acids also increased in these animals.

TABLE 1

Effect of restricted and ground roughage feeding on various physiological constituents of cows and goats

	Milk fat	Blood		Rumen acids		
		Glucose	Ketones	Acetic	Propionic	Butyric
	(%)	*(mg %)*		*(meq/liter)*		
4 Cows normal-fed	4.6	47.0	4.6	45.2	15.7	13.6
Restricted roughage	2.1	48.3	2.2	42.6	20.6	10.8
Difference	−2.5	+1.3	−2.4	−2.6	+4.9	−2.6
LSD (.05)[a]	0.3	3.0	0.8	5.4	3.6	2.0
4 goats normal-fed	3.3	54.2	2.9	42.4	11.0	9.4
Restricted roughage	2.6	54.5	1.5	45.1	25.4	8.6
Difference	−0.7	+0.3	−1.4	+2.7	+14.4	−0.8
LSD (.05)[a]	0.5	2.9	0.3	6.4	2.5	1.5
2 Goats normal-fed	2.9	53.6	2.8	37.9	9.3	8.7
Ground roughage	3.0	52.6	2.2	60.8	18.1	11.1
Difference	+0.1	−1.0	−0.6	+16.1	+8.8	+2.4
LSD (.05)[a]	1.2	5.3	0.6	11.9	3.7	1.9

[a] Least significant difference at 5% level of probability.

TABLE 2
Correlations between rumen acids, blood constituents, and milk fat per cent

	Cows Normal-fed df 19	Cows Fat-depressed df 59	Goats Normal-fed df 28	Goats Fat-depressed df 35
Rumen butyric × rumen propionic	+0.91**	+0.18	+0.83**	+0.09
Rumen butyric × rumen acetic	+0.78**	+0.76**	+0.85**	+0.60**
Rumen butyric × blood ketones	+0.72**	+0.35**	+0.06	+0.44**
Rumen butyric × milk fat	−0.28	−0.05	−0.21	+0.26
Rumen butyric × blood glucose	−0.37	−0.20	−0.39*	−0.17
Rumen propionic × rumen acetic	+0.82**	+0.56**	+0.91**	+0.14
Rumen propionic × blood ketones	+0.60**	−0.19	+0.30	−0.48**
Rumen propionic × milk fat	−0.31	−0.53**	−0.04	−0.51**
Rumen propionic × blood glucose	−0.14	+0.06	−0.21	−0.09
Rumen acetic × blood ketones	+0.37	+0.17	+0.12	+0.58**
Rumen acetic × milk fat	−0.37	−0.12	−0.14	+0.49**
Rumen acetic × blood glucose	−0.03	−0.24	−0.24	−0.04
Blood ketones × milk fat	−0.16	+0.10	+0.04	+0.71**
Blood ketones × blood glucose	−0.44*	−0.20	+0.20	+0.09
Blood glucose × milk fat	+0.42	+0.10	+0.18	−0.09

* Significant at the 5% level of probability.
** Significant at the 1% level of probability.

Other constituents that show significant positive relationships with milk fat are rumen acetic acid and blood ketones in milk fat–depressed goats. These correlations in the cow data are quite insignificant and may reflect a difference in metabolism between these species. A similar case is the apparent ketogenicity of acetic acid in the goats. There is a tendency for the correlation relationships to change in the animals on the test rations. In the cases cited, significance appears, and would suggest that some factor is making its appearance that might be related to the milk-fat depression. As has been already shown, there is a significant increase in rumen propionic acid and a significant decrease in blood ketones, on restricted roughage rations. In regard to the ketones, rumen butyric acid appears distinctly ketogenic (Table 2), whereas the relationship of ketones to propionic acid is not consistent.

The correlations between the rumen acids generally are very high. These high correlations are indicative of the dilution effects of feed, water, and saliva, as well as of the over-all fermentation, and are to be expected as a result of these effects. Marked exceptions to the general relationships of the rumen acids are that between propionic and butyric and, to a lesser extent, that between propionic and acetic, when the cows and goats were fed on restricted roughage. The lowered relationships with propionic acid in milk fat–depressed animals reflect how much the propionate fermentation is increased relative to the other rumen acids. Consequently, it would be predicted that the over-all effect will mask the true relationships that might occur between the rumen acids and blood constituents or milk fat. The effect may explain some of the anomalous correlations observed; for example, that between rumen propionic acid and blood ketones.

The extent to which these data can be reconciled to the reported antiketogenicity of propionate (6, 9, 19, 20) is demonstrated in the calculation of partial

correlations. The partial correlation for rumen propionic acid is -0.347^{**} with ketone bodies and -0.560^{**} with milk fat in fat-depressed cows when rumen acetic acid is held constant. Thus, propionic acid appears to have an exceedingly great antiketogenic effect, and bears highly significant negative relationships with milk fat, in milk fat–depressed cows and goats.

Thus, the results presented here are in essential agreement with other work on the metabolic effects of the addition of these fatty acids to the rumen or the injection of them into the blood (6, 19, 20).

Experiment 2. In view of the fact that McClymont (12) has already postulated that acetic acid is not the key factor in milk-fat depression, and that the results of the first experiment rather tended to support this view, an additional experiment was performed to compare—in normal lactating cows and milk fat–depressed cows—the arteriovenous differences in blood acetic acid and ketones.

Two groups of five cows each were used. In the first group, two Guernseys (68G and 116G) and three Holsteins (421, 424, and 443) were maintained for a time on a normal ration consisting of hay ad libitum plus 8 lb. of concentrates per day. Cows 421 and 424 were then placed on a ration of approximately 25 lb. of concentrates, plus about 7 lb. of alfalfa meal per day, while Cows 68G, 116G, and 443 were fed a ration of alfalfa meal ad libitum plus 8 lb. of concentrates per day. Triple blood samples (arterial, jugular, and mammary) were taken twice during the control period and twice during the experimental feeding period after the fat test had declined. The venous and arterial samples were drawn rapidly, with the least possible amount of excitement. The arterial sample was taken from the internal iliac artery via rectal puncture.

In Table 3 are data on the levels of arterial, mammary-venous, and jugular-venous acetic acids and ketone bodies of normal lactating cows and of cows fed restricted or ground roughage. There was a decrease of arterial acetic acid from the normal in fat-depressed cows and the apparent decrease of either the mammary or venous levels was slight. Thus, it appears in the arteriovenous differences that the mammary gland as well as the peripheral tissues are utilizing less acetic acid under conditions of milk-fat depression. The udders of milk fat–depressed animals appear to utilize proportionally slightly less of the available blood acetic acid (39% vs. 45% in controls). Smaller amounts of the ketone bodies appear to be utilized in milk fat–depressed animals. In these data, the mammary and the jugular venous levels have declined in milk-fat depression also, to about the same extent.

Experiment 3. To test the possibility that milk of low-fat content might be the result of large amounts of propionic acid produced in the rumen, a series of trials was run feeding sodium acetate and sodium propionate to cows with low milk fat on restricted roughage diets. The cows were fed salts mixed with the rations during five-day periods. Sodium acetate trihydrate was fed at the rate of 1 lb. per day and sodium propionate at the molecular equivalent of 300 g. per day. Ten successful trials were run with sodium acetate and six with sodium propionate, utilizing the same cows. All trials were rejected in which the cow decreased consumption of the ration containing the salts. In some of the pro-

TABLE 3

Arteriovenous levels of acetic acid and ketone bodies in milk fat–depressed cows

Treatment	Cow	Blood acetic acid						Blood ketone bodies					
		Concentration *(mg.%)*			Arteriovenous difference *(mg.%)*		Ratios of the differences	Concentration *(mg.%)*			Arteriovenous difference *(mg.%)*		Ratio of the differences
		Arterial	Mammary	Jugular	Mammary	Jugular		Arterial	Mammary	Jugular	Mammary	Jugular	
Normal-fed	68G	21.4	6.1	10.5	14.7	10.9	1.3	2.5	1.7	2.1	0.8	0.4	2.0
	116	17.1	6.9	10.5	10.2	6.6	1.5	3.7	2.3	2.7	1.4	1.0	1.4
		21.0	9.8	12.6	11.2	8.4	1.3	3.8	2.2	2.9	1.6	0.9	1.8
	421	14.1	9.5	12.7	4.6	1.4	3.3	3.3	2.2	2.7	1.1	0.5	2.0
		11.6	9.3	8.8	2.3	2.8	0.8	3.4	2.1	3.1	1.3	0.3	4.0
	424	15.4	8.2	9.2	7.2	6.2	1.2	2.9	1.8	2.3	1.1	0.6	1.7
		20.1	11.2	18.6	8.9	1.5	5.9	2.3	1.5	2.1	0.8	0.2	3.9
	443	21.0	9.8	12.6	11.2	8.4	1.3	2.6	1.9	2.2	0.7	0.4	2.0
		17.2	14.1	15.7	3.1	1.5	2.1	2.6	1.7	2.3	0.9	0.3	3.7
	Average	17.6	9.5	12.4	8.1	5.2	1.6	3.0	1.9	2.5	1.1	0.5	2.2
Milk fat–depressed	68G	15.6	9.6	11.9	6.0	3.8	1.6	1.8	1.2	1.6	0.6	0.2	3.2
		.....	.....	.....	.....	.....	....	2.1	1.8	1.7	0.8	0.4	2.1
	116	14.8	11.2	11.4	3.6	3.4	1.1	2.9	2.0	2.5	0.9	0.4	2.7
		.....	.....	.....	.....	.....	....	2.2	1.3	1.9	0.9	0.3	3.1
(Ground and restricted roughage diets)	421	11.1	3.6	7.6	7.5	3.5	2.1	2.9	2.5	2.4	0.4	0.5	0.9
		13.9	10.8	12.6	3.1	1.3	2.4	3.4	2.4	2.6	1.0	0.8	1.2
	424	11.8	6.4	7.8	5.4	4.0	1.3	1.6	1.0	1.4	0.6	0.2	2.3
		13.3	10.5	11.0	2.7	2.3	1.2	2.9	1.3	2.2	1.6	0.7	2.3
	443	16.1	5.5	7.3	10.7	8.8	1.2	1.4	0.8	1.2	0.6	0.2	2.9
		15.3	11.1	11.3	4.2	4.0	1.1	2.1	1.3	1.5	0.8	0.6	1.4
	Average	14.0	8.6	10.1	5.4	3.9	1.4	2.3	1.5	1.9	0.8	0.4	1.9

pionate trials it became necessary to administer the salt by drenching. The results are shown in Figure 1. Acetate caused a considerable increase in milk fat with a sizeable carry-over effect. Sodium propionate tended to cause a decline in milk fat per cent that recovered soon after feeding of the salt was stopped. Milk fat production paralleled the percentage, but included a factor of lactation decline.

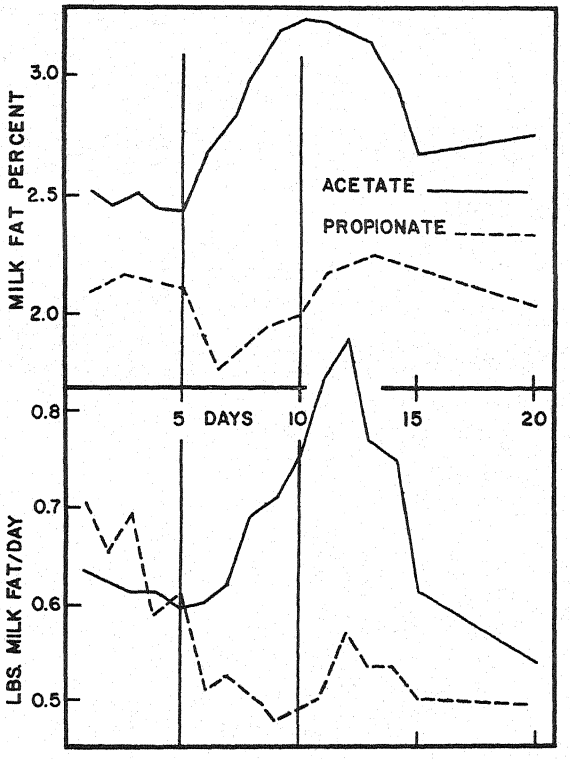


FIG. 1. Effect of sodium acetate and propionate on milk fat of cows fed restricted roughage. Sodium acetate trihydrate (450 g/day) and sodium propionate anhydrous (300 g/day) were fed between days five and ten.

DISCUSSION

The results on rumen concentrations do not seem to support the theory of a deficient supply of acetic acid, as such, on the part of the rumen organisms under conditions of ground and/or restricted-roughage feeding. It should seem that the molar proportion of rumen acetic acid decreases not as a result of a decreased acetate production but rather because of an increased propionate fermentation, as is shown in Table 1. This is the conclusion of Maki (10), who has indicated that there is an increase in numbers of bacteria that produce propionate, lactate, succinate, and acetate on restricted-roughage feeding. It was suggested that in isolated cases of milk-fat depression, the acetate fermentation is actually increased, but not nearly to the extent of production of propionate.

On the other hand, the results from the blood studies would indicate that acetate available to the mammary gland might be reduced. The reduction of rumen volume (1) on high-concentrate diets might be a factor involved. However, that acetate is not the only factor involved is suggested by the effects of feeding sodium propionate to cows producing low-fat milk, the effect being opposite that of feeding sodium acetate. In addition, levels of rumen propionic acid are negatively correlated with blood ketones, BHBA being a known milk fat precursor.

From the present knowledge of propionic acid metabolism, it is not difficult to find in it a common cause that will account for most of the conditions observed in milk-fat depression. The strong antiketogenic properties of propionate are well established (7, 9, 14, 19, 20). Pennington (14) describes the manner in which ketone-body formation may be inhibited by propionate. Propionate is converted into succinate and eventually oxalacetate, with which condensation with acetyl-CoA would divert the latter away from acetoacetate formation toward oxidation in the Kreb's cycle.

Thus, it is possible that an increase in the propionate-acetate ratio might divert the pathway of acetate metabolism away from synthesis of ketone bodies or milk fat, and provide over-all a lowered utilization of acetate. Both acetate and propionate are bound by CoA and competition for the enzyme is possible. This hypothesis would place increased emphasis on the importance of the ratio of rumen acids and is in harmony with the effects of feeding acetate and propionate salts to cows producing low milk fat.

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REPLACEMENT THERAPY IN LACTATING THYROPARATHYROIDECTOMIZED RATS[1]

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SUMMARY

Thyroparathyroidectomy was performed in 58 albino rats at Day 4 of lactation. The mother rats lost considerable body weight after the operation due, at least partially, to a reduced food intake. Replacement therapy was started immediately after operation. All animals received daily 3.5 μg. l-thyroxine/100 g. body weight and graded amounts of parathormone (PH) until Days 13 and 14 of lactation, respectively.

Based on the litter weight increase, daily injections of ten, 2 × 20 and 3 × 20 U.S.P. units of PH did not restore lactation to average control levels, whereas seven animals with presumed accessory parathyroid tissue reached normal or better than control levels. However, based on the weight of milk obtained on Day 14 of lactation under standardized conditions, expressed as per cent of the litter body weight as a criterion, 3 × 20 U.S.P. units PH/day resulted in successful replacement therapy in ten animals. Many incidents of tetany were recorded after discontinuing the PH supply and also in excellent lactating animals which were given 2 × 20 U.S.P. units PH/day. The withdrawal of PH on Day 13 resulted on Day 14 of lactation in a reduced blood calcium level, tetany, nervousness, depressed food intake and body weight, irregular nursing, disturbed lactogenic hormone discharge from the pituitary gland, presumably in an interruption of the let-down mechanism, and in markedly reduced milk yields.

The role of the parathyroid gland and its hormone, parathormone (PH), has been recognized for many years as playing a part in the incidence of parturient paresis (milk fever) in cattle, coincident with a reduction in blood Ca and P following parturition (2, 3, 23, 26). However, with Ca replacement therapy the symptoms are usually quickly alleviated and lactation continues. It has been assumed that the cause of this condition is a transient lowering of blood Ca and P following parturition, as intense milk secretion is initiated before the parathyroid glands are prepared to mobilize the Ca and P required for intense lactation. The question of the role of the parathyroid glands during normal lactation has received scant attention. It is possible, however, that parathyroid hormone (PH) secretion may be a factor which might tend to limit the secretion of milk throughout the lactation period (25). That the hormone is essential for normal lactation has been indicated, but no one has maintained normal lactation in parathyroidectomized animals (1, 6, 7, 8, 10, 11).

In the removal of the parathyroid glands in the rat, it is necessary to remove the thyroid glands also, due to their location (24). Lactation replacement thereapy, therefore, involves both thyroxine and the parathyroid hormone. It might be pointed out in passing that in previous studies of PH replacement,

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excessive amounts of thyroxine were administered (9). The hyperthyroidism so induced would have had a depressing effect on lactation even though parathyroid hormone replacement level was adequate. In the present study an optimal level of thyroxine was injected with graded levels of PH to determine the optimal level of PH for normal lactation in the albino rat.

MATERIALS AND METHODS

Primiparous lactating rats of the Sprague-Dawley-Rolfsmeyer strain were housed in individual cages, fed Purina Lab Chow and water ad libitum. The animal room was maintained at a uniform temperature of 78 ± 1° F. Shortly after delivery each litter was reduced to six young. Thyroparathyroidectomy was performed on Day 4 of lactation under combined Nembutal and ether anesthesia by a technique slightly modified from that of D'Amour and Blood (5). Care was taken that all visible thyroid and parathyroid tissue was removed and that the recurrent laryngeal nerve was not disturbed. In some groups, the completeness of thyroidectomy was checked by injection of 2 μc. I^{131} intraperitoneally, immediately after surgery. The 24 hr. uptake of I^{131} in the thyroid region was used as a criterion of completeness of thyroid removal. Replacement therapy was started shortly after operation. All animals were injected uniformly with 3.5 μg. l-thyroxine/100 g. body weight and graded amounts of PH[3] (Table 1). Milk secretion was measured by the increase in mean litter weight to Day 14. As a further check on lactation intensity, the amount of milk removed by the litter on Day 14 was estimated by the method described by Grosvenor and Turner (13). Milk was weighed on a balance accurate to 0.1 g. The mother rats were killed by etherization, then decapitated for blood collection when serum determinations were made. Serum calcium was determined by the method of Clark and Collip (4), as described by Harrow and Alii (22). The pituitaries were removed rapidly, weighed on a micro-balance accurate to 0.05 mg., and frozen for lactogenic hormone assay (16).

[3] Supplied by Eli Lilly and Company, Indianapolis, Ind.

TABLE 1

Replacement therapy with thyroxine and graded levels of parathyroid hormone

Daily replacement therapy[a] parathyroid hormone	No. of mothers			No. of young of surviving mothers		
	Day 4 p.p.	Dead with tetany during experiment	Day 14 p.p.	Day 4 p.p.	Dead during experiment	Day 14 p.p.
No U.S.P.[b]	10	2	8	48	4	44
10 U.S.P.	10	1	9	54	13	41
2 × 20 U.S.P.	10	3	7	42	1	41
3 × 20 U.S.P.[c]	11	1	10	60	. .	60
Accessory glands(?)	7		7	42	1	41

[a] All rats received 3.5 μg. thyroxine/100 g. body weight daily from the fourth to the 13th day of lactation by subcutaneous injection. The rats in Group 3 × 20 received the first injection 24 hr. after operation at Day 5 p.p., the next injection at Day 7 p.p.

[b] The parathyroid hormone was standardized in terms of U.S.P. units. It was injected subcutaneously from Day 4 to Day 13 of lactation, in the 3 × 20 group to Day 14.

[c] Two microcuries I^{131} immediately after operation.

RESULTS

The mother rats showed losses in body weight after the operation until Day 9. They recovered, but did not show final weight gain equal to normal rats (Table 2). The group receiving 3 × 20 U.S.P. units was given 2 × 30 U.S.P. units PH on Day 14 previous to the lactation test. This resulted in normal behavior and normal food intake, in contrast to the other groups in which PH replacement therapy was not given or was discontinued on Day 13. Hormone withdrawal on Day 13 increased nervousness and depressed food intake and body weight on Day 14.

The average weight gain of the litters remained below the figures for control animals (Table 3); whereas, 10 U.S.P. units PH/day were ineffective, 2 × 20 and 3 × 20 U.S.P. units PH/day, respectively, showed fairly successful replacement therapy. However, the withdrawal of the 10 and 2 × 20 U.S.P. units PH/day, respectively, on Day 13 resulted in a sharp drop of the growth curve. This was not observed in the seven animals with presumed accessory parathyroid tissues which reached practically control levels. There is no explanation for the drop in the growth curve of the animals which received no parathormone replacement therapy (Figure 1).

TABLE 2

Body weight of mother rats

Daily replacement therapy parathyroid hormone	Average body weight of surviving mothers during lactation						Average body weight loss of surviving mothers between Day 4 and Day 14
			Day 14				
				Evening			
	Day 4	Day 9	Morning	Pre-nursing	Post-nursing		
	(g.)						(%)
No U.S.P.	270.9	248.6	249.9	241.9	240.4	30.5	11.0 ± 3.2
10 U.S.P.	285.1	243.2	252.4	257.2	254.1	31.0	10.8 ± 2.1
2 × 20 U.S.P.	289.7	265.1	266.6	263.3	259.7	30.0	10.2 ± 1.5
3 × 20 U.S.P.	275.6	262.6	266.6	278.6	271.0	4.6	1.7 ± 2.6
Accessory glands (?)	262.0	253.7	263.4	261.6	252.0	10.0	3.6 ± 1.7
Controls n: 35	291.97			300.03		+8.0	+3.2 ± 1.5

TABLE 3

Average litter weight gain between Day 4 and Day 14 of lactation

Daily replacement therapy parathyroid hormone	Day of lactation		Weight gain
	4	14	
	(g.)	(g)[a]	(%)
No U.S.P.	56.0	93.1	67.2 ± 16.5
10 U.S.P.	58.9	85.2	45.9 ± 19.0
2 × 20 U.S.P.	58.9	137.1	133.5 ± 16.4
3 × 20 U.S.P.	56.7	138.8	145.5 ± 6.9
Accessory glands (?)	56.9	156.3	175.0 ± 9.7
Control n: 35	58.8	172.7[b]	194.5 ± 3.5[b]
Control[c] n: 30		165.6	

[a] Corrected for weight of milk obtained during 30 min. of nursing at Day 14.
[b] Noncorrected for milk obtained during 30 min. of nursing.
[c] Data from Grosvenor and Turner (20): 30 rats, 3 μg/100 g/day l-thyroxine injected subcutaneously, Days 7-13.

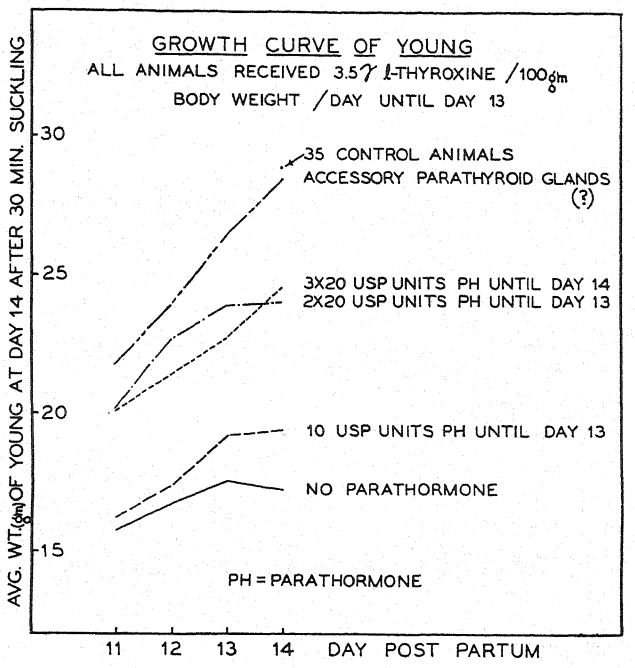


FIG. 1. Average weight of young at Day 14 after nursing. The withdrawal of parathormone (PH) at Day 13 in groups on ten and 2 × 20 U.S.P. units PH, respectively, resulted in a drop in the growth curve of young. This was not observed in the young of mothers believed to have accessory parathyroid tissue.

In all groups, incidences of tetany were observed, except in those animals which have been considered to have accessory parathyroid tissue (Table 4). The better the lactational performance due to replacement therapy, based on the litter weight increase, the more severe and more frequent became the attacks of tetany, especially when 2 × 20 U.S.P. units PH were given daily. Only one rat which received 3 × 20 U.S.P. units PH died in a fatal attack of tetany, after having been the best lactator in this group, as indicated by litter weight gain.

TABLE 4

Incidence of tetany

Daily replacement-therapy parathyroid hormone	Days post partum										
	4	5	6	7	8	9	10	11	12	13	14
No PH			TT								
10 U.S.P.				T							tT[a]
2 × 20 U.S.P.			t	tT	T	T				t	T[a]
3 × 20 U.S.P.					t	t		t[b]		T	
Accessory glands (?)											

T: fatal.
t: animal survived.
[a] Heavy attack of tetany during nursing.
[b] Second attack of the same animal.
In all cases when tetany was recorded, 20 to 30 U.S.P. units PH were given immediately, intraperitoneally and subcutaneously.

On the other hand, the withdrawal of parathormone on Day 13 resulted in many cases of tetany on Day 14 in the groups which received 10 and 2 × 20 U.S.P. units PH daily.

In the group on 3 × 20 U.S.P. units, certain animals had a pulmonary condition which interfered with lactation, as indicated by reduced litter growth. These animals were discarded, but it is interesting to note that tetany also occurred in many of these animals after the withdrawal of PH.

In the groups on 10 and 2 × 20 U.S.P. units PH replacement therapy, treatment was discontinued on Day 13 and the lactation test described by Grosvenor and Turner (13) conducted on Day 14. The withdrawal of hormone on Day 13 increased the nervousness and depressed the food intake. As a result, the milk yields were low. In contrast, the group on 3 × 20 U.S.P. units received 2 × 30 U.S.P. units PH on Day 14. In spite of the fact that litter weight increase was almost the same as in the 2 × 20 U.S.P. units group, the mean milk yield exceeded that of control animals. The animals believed to possess accessory parathyroid glands (had received daily no, ten, and 2 × 20 U.S.P. units PH, respectively, plus 3.5 μg. l-thyroxine/100 g. body weight) showed the highest mean milk yield, although the PH, if given, was discontinued on Day 13.

Many of the mother rats receiving no PH or 10 and 2 × 20 U.S.P. units PH/day, respectively, until Day 13 were unable to give any milk, although the young had been suckling continuously.

The stimulus of nursing causes a release of oxytocin which facilitates the removal of milk and simultaneously causes the discharge of lactogenic hormone (12, 17). These effects can be blocked by Nembutal anesthesia (15). The poor milk yields on the lower levels of pH and after its withdrawal on Day 13 suggested that calcium deficiency and resulting effects on the nervous system might interfere with the normal transmission of the nursing stimuli and subsequent oxytocin and lactogen release. Data are not available on the release of oxytocin in these animals at the time of nursing. However, the pituitaries of the various groups were assayed for lactogenic hormone (16). Since the pituitaries were obtained after nursing, they would be expected to show low post-nursing levels of lactogenic hormone if normal release had occurred (17). However, as it is shown in Table 5, only the animals receiving 3 × 20 U.S.P. units of PH until Day 14 show a normal post-nursing level; whereas, groups on 10 and 2 × 20 U.S.P. units PH until Day 13 indicate an elevated concentration ranging between pre- and post-nursing levels. The animals which did not receive pH indicated that very little lactogenic hormone discharge had occurred during the nursing period. The gradual decline of lactogenic hormone concentration combined with an increasing yield of milk is believed to indicate that replacement therapy and more nearly normal blood Ca levels permitted improved transmission of the nursing stimuli to lactogen and oxytocin release.

However, no explanation can be given for the high post-nursing lactogenic hormone concentration in the pituitaries of the seven animals with presumed accessory parathyroid tissues. A similar phenomenon was described by Grosvenor and Turner (17) at Day 21 of lactation in intact animals.

TABLE 5

Lactational performance and lactogen content of the pituitary

Daily replacement therapy parathyroid hormone	Weight of milk	Weight of milk / Weight of litter	Mean weight of pituitary		I.U. lact. H/g pituitary	I.U. Lact. H/100 g. rat post-nursing B.W.
				mg/100 g. Post-nursing body weight		
	(g.)	*(%)*	*(mg.)*			
No U.S.P.	1.6 ± 0.4	1.7 ± 0.3	9.1	3.8 ± 0.1	73.8	0.282
10 U.S.P.	2.9 ± 0.8	3.0 ± 0.8	9.7	3.8 ± 0.1	32.0	0.110
2 × 20 U.S.P.	3.6 ± 1.1	2.4 ± 0.6	10.7	4.1 ± 0.1	22.9	0.094
3 × 20 U.S.P.	7.5 ± 0.7	5.3 ± 0.5	10.4	3.8 ± 0.1	4.0	0.016
Accessory glands (?)	9.5 ± 0.4	5.9 ± 0.2	10.4	4.1 ± 0.1	50.0	0.209
Controls						
[a]			10.2	3.5		
[b]	5.5	3.8 ± 0.2				
[c]	6.8	4.3 ± 0.4	9.3	3.2	94* 10**	
[d]	6.2	3.8 ± 0.3		3.4		
[e]	9.5	5.2 ± 0.2		3.7		

[a] (12).
[b] (14).
[c] (17).
[d] (19).
[e] (20) 30 rats, 3 μg/100 g. body weight/day 1-thyroxine, subcutaneously.

* Pre-nursing concentration.
** Post-nursing concentration.



Since it was observed that the young of rats which suffered tetany while nursing were unable to obtain milk, preliminary serum calcium determinations were made to determine if there was a relation between serum calcium level and the lactational performance. Two groups of ten lactating rats were injected with 3×20 U.S.P. units PH daily after thyroparathyroidectomy. In one group, the PH injections were discontinued 24 hr. before the end of the experiment. The second group was injected with 2×30 U.S.P. units PH on Day 14 during the last 12 hr. of the experiment. After withdrawal of PH on Day 13, the serum Ca level dropped within 24 hr. to 7.95 ± 0.51 mg. %. In the second group in which PH was given until the end of the experiment, serum Ca level was 11.45 ± 0.39 mg. %. This observation supports the suggestion that withdrawal of the parathormone on Day 13 by reducing the blood calcium level influences the neural transmission of the nursing stimuli.

The ability of the thyroid gland to concentrate I^{131} was used as a criterion to check the completeness of the removal of thyroid tissue. The 24-hr. uptake of I^{131} by the thyroid gland of intact animals of this strain of rats was between 10 and 20% of the injected dose. In the thyroparathyroidectomized animals the 24-hr. uptake of I^{131} injected immediately after surgery was only $0.76 \pm 0.25\%$. Since I^{131} is excreted in the urine, the bladder of these animals was checked simultaneously and $2.89 \pm 0.96\%$ of the injected dose was detected. These data lead to the conclusion that no appreciable amount of thyroid tissue was left in the neck region.

DISCUSSION

Replacement therapy has not yet been achieved in the thyroparathyroidectomized lactating albino rat. For such replacement, it is necessary to provide not only optimal amounts of parathormone but of thyroxine as well. Recently, in this laboratory, the variation in thyroxine secretion rate (TSR) of this strain of lactating rats has been determined. The mean TSR was 2.2 μg per 100 g. body weight, with maximum rate of 3.5 μg. (18, 19). Optimal replacement of thyroxine should result at this level. The effectiveness of 3.0 μg./100 g. body weight administered to intact lactating rats was shown to increase their milk production by 37% (20). With this information at hand, the determination of the parathormone requirement during lactation should be facilitated.

The rats were thyroparathyroidectomized on Day 4 of lactation. Without parathormone replacement (thyroxine given) the increase in litter weight was minimal, indicating poor lactation capacity. As the parathormone was increased to ten there was a slight increase, and with 2×20 U.S.P. units, a marked increase in litter weight, although still considerably below normal. It was hoped that the increase to 3×20 U.S.P. units would further improve mean litter weight. In this we were disappointed, for litter weight was not increased. That the higher level was effective in maintaining more nearly normal Ca levels is indicated by the absence of tetany, except in a single animal, compared to more frequent tetany in the group on 2×20 U.S.P. units.

It is possible that surgery on Day 4 of lactation is a sufficient stress on the mothers to interfere with lactation and impede litter growth, which can not be

recovered during the test period. The evidence for the rate of lactation on Day 14 should be of interest in connection with this problem. In the first groups, the hormone was discontinued on Day 13. The milk yields on Day 14 were seriously influenced by this withdrawal. The observations obtained, however, were of great interest, for they showed that the hormone's action is of short duration for serum Ca decreased, incidence of tetany increased, and the nervous mechanism of oxytocin and lactogen discharge impeded.

In the group given 3×20 U.S.P. units up to and including Day 14, the mean milk yield was 7.46 ± 0.72 g., which surpassed the control milk yield but did not equal the milk yield of normal animals given thyroxine. But, since the body weight of the young is somewhat lower in this study, the values for the amount of milk divided by the weight of litters reaches almost the same level found by Grosvenor and Turner (20) in intact rats, given 3.0 μg. l-thyroxine/100 g. body weight/day to improve the milk yield.

The fact that 3×20 U.S.P. units per day, while not stimulating normal litter growth, appeared to control tetany (except in one intense lactator), and on Day 14 produced a mean milk yield approaching that of normal rats given thyroxine, is believed to indicate that this level of parathormone therapy is close to the optimal level.

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A CRITICAL STUDY OF ENERGY DETERMINATION IN FRESH AND DRIED COW FECES [1]

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SUMMARY

An accurate method of determining energy in fresh feces is described in detail. The energy content of eight samples of cow feces was determined in freeze-dried, oven-dried, and fresh feces. Moisture was determined by freeze drying, by the toluene distillation method, and by oven drying at 80° C. The three methods are compared and discussed.

The results of the duplicate energy determinations in the feces dried by two different methods, and the triplicate determinations in the fresh feces calculated on a comparable basis by means of the three different moisture determinations, are compared and evaluated and their errors discussed. The difference between the energy content of freeze-dried, oven-dried, and fresh feces calculated to a comparable basis by means of the most reliable water determination used in this study (freeze drying) was less than 2%.

Two papers (2, 6) have been published recently dealing with the determination of energy in fresh cow feces employing a combustion primer. These investigators used ethanol to promote combustion and found energy values averaging 13.8% (range—4.1–20.9%) and 3.3 ± 3.1% higher than when the same material was oven-dried and then burned in the usual way. The lower values in the oven-dried material were attributed to fermentation and loss of ammonia and other volatile substances during the drying process.

The first investigators (2) worked with feces having a dry matter content of 20–25%. The average dry matter content of cow feces collected in recent digestion trials at this station has been approximately 12% (range 10–14%). Complete combustion of such wet feces was found to be impossible when 1 ml. of ethanol was added to a 2-g. sample. The senior author of this paper developed a new technique in 1956 to overcome this difficulty and has made a thorough, detailed study of the factors which affect the determination.

EXPERIMENTAL PROCEDURE

The first step was an attempt to secure complete combustion by mixing ethanol with feces, using a short piece of glass rod (3 cm. long by 0.35 cm. diameter), the rod being left in the combustion cup during the determination. Control tests with benzoic acid showed no change in energy value due to inclusion of the glass rod. Complete combustion was attained by this procedure, but it at once posed another problem. The mixing process resulted in volatilization of a considerable amount of ethanol, which gave lower results and also made it very difficult to obtain triplicate determinations within an acceptable range of variation. The error was further compounded by the high ratio of the energy in the added ethanol to the energy in the sample of fresh feces (approximately 5 or 6 : 1). For these reasons the use of ethanol as a primer was abandoned.

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Search for a suitable substitute resulted in the choice of dimethylformamide. This organic solvent has a boiling point of 153° C. and has the following advantages: (a) Loss due to volatilization during preliminary manipulations apparently approaches zero; (b) it can be weighed on an analytical balance instead of being measured by a pipette, thus contributing to greater accuracy; (c) slow combustion results in a slow and uniform rise in temperature in the adiabatic calorimeter, an especially desirable feature.

The method in this final form has been used for the energy determinations in fresh feces, reported in this paper. Energy was determined also on samples of feces which were dried in an oven at 80° C. with forced-air circulation, and by freeze drying. A separate duplicate water determination of a 3-g. sample of both types of dried, ground feces was made in an oven at 105° C. for 3 hr. at the same time. These results are used only for the calculation of the energy in the samples of dried feces to dry matter basis.

To compare the results of the energy determination of fresh feces with those for the dry feces, it is necessary to know the exact total water content of fresh feces. Therefore, the moisture content of the feces in the present experiment was determined in three different ways: (a) By the toluene distillation method, using 100-g. aliquots; (b) by oven drying at 80° C. with forced-air circulation; (c) by lyophilizing (freeze drying).

If in the Sterling-Bidwell toluene distillation method the 25-ml. capacity side-arm receiver (0.2-ml. divisions) is used, the amount of water can at best be read by estimate to only 0.1 ml. Conversion of such a reading to a percentage basis compounds any error fourfold. This means that a side-arm receiver of this capacity permits an accuracy of 0.5% on the wet basis. Converted to a dry basis any error can be compounded as much as eight times. Therefore, specially designed side-arm receivers of 100-ml. capacity graduated from 75–100 ml. in 0.1-ml. intervals were used in these experiments. For the freeze drying, 70-g. samples weighed to the nearest 0.1 g. were used and dried by the usual procedure, after some water was added to secure a more even distribution along the walls of the container.

Two of the quadruplicates of each sample were used for the water determination. After drying under high vacuum was completed, the bottles were immediately stoppered and weighed. To be sure that all water was removed, the bottles were placed in an oven at 105° C. for 3 hr., weighed again, and these final results used for the calculation of the water determination.

Although duplicate aliquots were used for the toluene distillation method and for the freeze-drying method, only a single sample of approximately 1,200 g. was used for oven drying. This represented the bulk of the ten-day composite from a digestion trial. The drying was done in shallow agateware pans (16 by 12 by 2½ in.) in a large thermostatically controlled oven specially designed for the rapid drying of large samples. The drying was accelerated by frequent stirring, to break the crust which formed, and was completed in about 6–7 hr.

RESULTS AND DISCUSSION

Gross energy values (by actual determination). A glance at the average values in Table 1 shows that there is a consistently higher energy content in the freeze-dried feces than in the corresponding oven-dried samples. This might be due to a greater loss of volatile compounds with relatively low energy values during the freeze-drying process than during oven drying. This would result in a small gain of energy per unit of weight.

Comparing the variation of the duplicate determinations of energy by both methods, very little difference is noted. The higher variation in the determinations of the freeze-dried feces reflects difficulty in handling the light, fluffy material resulting from this procedure. The variation among the triplicate determinations of the energy in fresh cow feces, which is five to six times higher than that obtained by freeze drying or oven drying, is caused by the heterogenous character of fresh cow feces and by the variation in the amount of primer which was added to start combustion. The blank deducted from gross energy of fresh feces plus primer is the average value of five determinations which had a standard variation of 0.21%. The normal energy ratio of this primer to fresh feces is 4 or 5 : 1. Therefore, a variation of the primer alone can result in a variation in energy value of the fresh cow feces of 0.8 to 1.0%.

Moisture determination. The results in Table 2 show that the dry matter values obtained by oven drying at 80° C. are consistently higher than those obtained by either the toluene or the freeze-drying method. Comparing the results of the freeze-drying and toluene methods, it must be stated that only half the values show any considerable difference. The average dry matter value for the toluene method is 1.33% lower, and for oven drying at 80° C. is 2.57% higher, than the average dry matter content of the lyophilized feces.[2] The consistently higher dry matter values of the oven-drying method at 80° C. are due to incom-

[2] This value equals 100%.

TABLE 1

Detailed gross energy values in cow feces as obtained by three methods

Cow No.	In freeze-dried feces			In oven-dried feces			In fresh feces[a]		
	Mean value of 2 determinations	Variation		Mean value of 2 determinations	Variation		Mean value of 3 determinations	Variation	
	(cal/g d.m.)	*(cal/g)*	*(%)*	*(cal/g d.m.)*	*(cal/g)*	*(%)*	*(cal/g fresh)*	*(cal/g)*	*(%)*
2	4,889.4	2.46	.05	4,853.6	22.30	.46	645.7	7.94	1.23
8	4,876.2	17.52	.35	4,846.8	5.29	.11	507.6	9.51	1.87
10	5,067.8	21.07	.42	4,988.8	8.60	.17	593.8	12.60	2.13
11	4,933.4	7.02	.14	4,855.8	.29	.01	715.6	4.57	.64
16	4,915.6	5.44	.11	4,880.1	13.33	.27	676.3	.90	.13
17	5,089.5	11.36	.22	5,004.7	5.48	.11	582.5	3.59	.62
20	4,955.2	2.83	.06	4,902.3	3.84	.08	716.2	4.42	.62
40	5,088.0	9.04	.18	4,993.8	1.68	.03	563.7	5.22	.93
Av.	4,976.9	9.59	.19	4,915.7	7.60	.16	625.2	6.09	1.02

[a] See Table 3 for energy values in fresh feces calculated to dry matter basis by using the results from the three methods of moisture determination as set forth in Table 2.

TABLE 2

Detailed moisture values in cow feces as obtained by three methods

Cow No.	Toluene method 100-g. sample: % Dry matter Mean value of 2 determinations	Toluene method 100-g. sample: Variation	Toluene method 100-g. sample: Variation in % of mean value	Freeze-drying 70-g. sample: % Dry matter Mean value of 2 determinations	Freeze-drying 70-g. sample: Variation	Freeze-drying 70-g. sample: Variation in % of mean value	Oven-dried at 80° C. 1,200-g. sample: % Dry matter One determination
		(%)			*(%)*		
2	13.35	.12	.90	13.51	.01	.07	13.43
8	10.16	.17	1.67	10.63	.04	.38	11.16
10	11.76	.31	2.64	12.12	.01	.07	12.46
11	14.77	.13	.88	14.76	.04	.27	15.06
16	14.05	.01	.07	14.04	.01	.07	14.60
17	11.66	.18	1.54	11.67	.05	.43	11.77
20	14.46	.01	.07	14.78	.15	1.01	14.98
40	10.96	.17	1.55	10.99	.16	1.46	11.64
Av.	12.64	.14	1.16	12.81	.06	.47	13.14

plete removal of the hygroscopic moisture. It will be shown (in Subsection 3 of Table 3) that this constant error results in somewhat lower calculated energy values in the dry matter, when the moisture factors thus obtained are applied to the energy values obtained with fresh feces. A comparison of the average variation of duplicate determinations of the toluene and freeze-drying methods (see Table 2) shows that the variation of the results of the toluene method is more than twice that of the average value of freeze drying, even though specially designed side-arm receivers were used.

Gross energy values (calculated from energy values of fresh feces and moisture determinations by the three different methods). Study of Table 3 shows that the method of moisture determination has a considerable effect on the final result, when the values determined in fresh feces are calculated to the dry basis, using the moisture factors from Table 2. Only the variation of the triplicate energy

TABLE 3

Calculated gross energy values[a]

Cow No.	Toluene method: Mean value of 3 determinations	Toluene method: Variation	Freeze-dried: Mean value of 3 determinations	Freeze-dried: Variation	Oven-dried at 80° C.: Mean value of 3 determinations	Oven-dried at 80° C.: Variation
	(cal/g d.m.)	*(cal/g)*	*(cal/g d.m.)*	*(cal/g)*	*(cal/g d.m.)*	*(cal/g)*
2	4,836.6	59.49	4,779.3	58.79	4,807.8	59.14
8	4,996.1	93.45	4,775.2	89.30	4,548.4	85.05
10	5,049.6	102.17	4,899.6	104.36	4,765.9	101.51
11	4,844.7	31.01	4,847.9	31.03	4,751.4	30.42
16	4,813.5	6.26	4,816.9	6.26	4,644.1	6.02
17	4,995.7	30.97	4,991.4	30.95	4,949.0	30.68
20	4,952.7	30.71	4,845.5	30.04	4,780.8	29.64
40	5,142.9	47.83	5,128.9	47.70	4,842.5	45.03
Av.	4,954.7	50.23	4,885.6	49.80	4,761.2	48.44

[a] Energy in fresh feces calculated to dry matter basis using the results of the three methods of water determination.

determination, not the influence of the variation of the duplicate water determination, is included in these figures. Therefore, the values for per cent variation are the same as those obtained from the values for fresh feces in Table 1.

The energy values of oven-dried feces were used for comparison with those from fresh feces calculated to the dry matter basis, using the results of the three different methods of water determination. Using the toluene method, the energy content of the calculated feces was 0.79% higher than that of the oven-dried feces; using the freeze-drying method and the oven drying at 80° C., these calculated values were 0.61 and 3.14% lower than the energy content of the oven-dried feces. This last high value reflects, as mentioned above, the incomplete removal of moisture in an oven at 80° C. According to these results, there seems to be no important difference between the energy content of oven-dried cow feces and the corresponding fresh feces burned by the aid of a primer (diamethylformamide) and calculated to a comparable basis by means of the moisture determination, using the Sterling-Bidwell toluene method or freeze drying.

These results are not in agreement with the findings of many researchers during the last 100 yr., whose interest was directed mainly to the loss of nitrogen during the oven drying of feces. A few of their results are listed in Table 5.

TABLE 4

Heat of combustion of some compounds in excrements which might be affected by the drying process

Name	Kcal/mol(13)	Mol. wt.(9)	Cal/g	Density g/ml(9)
Ammonia	55.01(18)	17.03	3,230.18	lq. 0.817[a]
Hippuric Acid	1,012.0	179.17	5,648.27	1.371
Urea	152.0	60.06	2,530.80	1.335
Uric Acid	460.20	168.11	2,737.49	1.893

[a] = −79° C.

TABLE 5

Nitrogen loss during drying of feces expressed as per cent of total N

Cow	Horse	Sheep	Hog	Rabbit
		(%)		
1.20–1,85 (8)	0.00 (22)	0.42 (24)	1.66– 2.03 (5)	0.55–5.56(7)
2.52 (24)	0.00–1.27 (21)	0.00–2.34 (15)	2.88 (24)	
3.00–4.50 (1)	2.56 (14)	2.66 (16)	2.28– 9.74 (3)	
4.86–6.70 (11)	4.43 (19)		2.79–10.34 (17)	
4.48–7.19 (12)	5.93 (24)		2.98– 9.85 (4)	
	0.00–6.44 (23)		8.06 (20)	
			Up to 10.00 (10)	

Most of these figures, of course, do not represent the nitrogen loss alone but are due in part to error in water determination. Doubtless any loss of organic material during the drying process will also result in too high a water value, which in the calculation on a comparable basis will cause an over-estimation of the nitrogen loss.

The same is true for the energy determination. The following general facts may be deduced for the energy loss during the drying process of feces:

If the energy value of 1 g. volatile organic material is higher than that of 1 g. residual dry matter, the energy content of 1 g. dry matter in fresh feces will be higher than that of 1 g. dry matter in the dried feces.

If the energy value of 1 g. volatile organic material is lower than that of 1 g. residual dry matter, the energy content of 1 g. dry matter in fresh feces must be lower than that of 1 g. dry matter in the dried feces.

If the energy value of 1 g. volatile organic material is the same as 1 g. residual dry matter, the energy content of 1 g. dry matter in fresh feces will be equal to that of 1 g. dry matter in the dried feces.

Kleiber (12), who investigated the loss of organic matter during drying, used a specially designed oven. He determined the carbon losses during the drying of cattle feces and found in 14 trials a loss of 1.36 ± 0.08% C of the total carbon content of feces; 45–65% of this C loss was combustible. Ghoneim (7), who also used a specially designed system found, in 11 trials with rabbit feces, an energy loss of 1.37%, ranging from 0.51 to 2.89% at a drying temperature up to 110° C.

Since Kleiber found that only about half of the lost carbon was combustible, and most of the nitrogen loss appeared in the form of ammonia (20) which has an energy value of only 3,230 calories per gram (Table 4), it must be assumed that the energy content of 1 g. of volatile organic material in fresh cow feces is smaller than that of 1 g. dried feces. According to the second statement above, this means that the energy content of 1 g. dry matter in fresh feces has a lower energy content than 1 g. dry matter in dried feces, or the displacement of volatile organic material of low-energy content per gram during the drying process has resulted in a small increase in energy content per gram dry matter.

The application of this point of view to the results of the experiment described above would mean that the calculated energy contents of fresh feces using the toluene method and the freeze-drying method and the energy value of the oven- and freeze-dried feces are too high. The actual energy value of 1 g. dry matter of fresh feces must be somewhere between the value obtained in fresh feces and calculated by the water determination at 80° C. and the energy content of oven-dried feces. A reliable figure is not available from research conducted thus far, because of the inaccuracy of the water determination, which is necessary for the calculation of the results for energy to a comparable basis. There is no method in general use which would exclude the volatile compounds from measurement as water.

Because this study shows that the difference between the energy content of oven-dried feces and fresh feces, calculated on a comparable basis by means of the freeze-drying water determination, is relatively small (averaging only 0.61%), it appears that the method used in this study to determine the energy content in fresh feces gives satisfactory results (variation range 1.0%). It is particularly useful in studies where the digestibility of protein and energy are the only values of interest.

In studies where the feces have to be dried and ground, it is much less time-consuming to obtain closely agreeing parallel results (variation range 0.2%) by determining gross energy in the dried and ground material. In such a case, more emphasis should be put on the accuracy of the water determination.

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TECHNICAL NOTES

COLD SHOCK OF EPIDIDYMAL SPERMATOZOA

Lasley and coworkers (3, 4, 5) reported that epididymal spermatozoa were resistant to cold shock and that ejaculated spermatozoa were not. They have likewise reported that ejaculated seminal plasma did not affect the cold shock response of epididymal spermatozoa and that as spermatozoa progressed from their place of origin outward they became more sensitive to shock. Bialy and Smith (1) reported that in the ductus deferens and ampulla of the bull there was not a sufficient number of spermatozoa to comprise an average ejaculate. Thus, it appeared that during their transition from the epididymis to the time that spermatozoa became a part of the ejaculate, the cells became susceptible to cold shock.

Spermatozoa for this study were obtained from slaughterhouse bulls. Testes and ampullae from the same bull were kept together. The time interval between the slaughter and the beginning of experimental work was approximately 2 hr. Spermatozoa from the cauda epididymis and the ampulla were washed out under pressure with a 2.9% sodium citrate solution, utilizing about 15 ml. of fluid. Ampullae from 13 bulls were utilized in the study of cold shock of ampullar sperm. The work with epididymal sperm involved 25 bulls. Experimentation was not undertaken until full motility of epididymal spermatozoa was visible. Evaluations of the per cent of motile sperm were made with the aid of the microscope. Cold shock consisted of placing 1-ml. volumes of suspended spermatozoa from room temperature (about 25° C.) into a bath at 0° C. for 10 min. The epididymal samples were treated as follows:

A—Fresh, untreated

B—Sample A, cold-shocked

C—Sample A, injected once through a corresponding ampulla from which endogenous spermatozoa previously were evacuated.

D—Sample C, cold-shocked.

Spermatozoa from the ampullae showed very little resistance to cold shock, with the untreated samples showing 76.5% motile spermatozoa and the cold-shocked only 23.3%. The differences among the epididymal samples were analyzed according to Snedecor (6) and Duncan (2). Comparison of the means is shown in Table 1.

The results obtained in this study follow closely the results obtained by previously mentioned workers (3, 4, 5). Histological examination of ampullar slices reveals a large number of interlocking channels which afford spermatozoa a large area of contact with the secretory epithelium of the ampullae. It appeared that during a normal ejaculatory process, spermatozoa from the cauda epididymis come in contact with secretions of the ampullae and are rendered susceptible to cold shock. The reason for the ineffectiveness of seminal plasma to render epididymal spermatozoa susceptible to cold shock may be twofold:

1. The factor in the ampullae becomes attached to spermatozoa and is not available in the plasma proper.
2. The factor either is unstable or is inactivated by other components of seminal plasma.

TABLE 1

Comparison of the mean per cent motility rating of the cauda epididymal spermatozoa

Treatment	A	C	B	D
Mean[a]	77.5	76.2	70.7	42.0

[a] Means not underlined by the same line are significantly different at the 1% level of probability.

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SOURCE OF IODINE IN SALT AFFECTS PROTEIN-BOUND IODINE CONTENT OF BOVINE BLOOD PLASMA[1]

During June, 1958, to May, 1959, blood plasma samples of approximately 350 dairy calves, heifers, and cows in the College herd were analyzed for protein-bound iodine (PBI) by the Brown method, as modified by Lennon and Mixner (2). The PBI values ranged from 2 to 42 γ per 100 ml., with about 15% of the samples above 20, and 75% above 10 γ per 100 ml. Asplund *et al.* (1) of West Virginia have reported that levels of PBI from 15 to 30 γ per 100 ml. were common in their study, and values as high as 75 γ per 100 ml. were obtained. Both of the observations were distinctly higher than those found in other laboratories. In a search for an explanation of the high values, the iodine in the trace-mineralized salt was considered worthy of checking. Long *et al.* (3) found no effect when 22 mg. of potassium iodide were fed daily, but 44 mg. caused an increase in the PBI of dairy cows. Calculated intakes of iodine from the 1% of trace-mineralized salt in the concentrated feeds in our studies ranged from 2.1 to 5.2 mg. per cow daily. In addition, blocks of trace-mineralized salt were available to the animals of the herd.

To explain the high values of iodine in these studies, an experiment was designed in which the 1% trace-mineralized salt in the concentrate mixture was replaced for 2 wk. with plain salt for six heifers averaging 6 mo. of age. The results are presented in Table 1. The heifers averaged 19.55 PBI γ per 100 ml. at the beginning of the experiment while trace-mineralized salt was fed; 3.78 γ per 100 ml. after removal; and 10.87 γ per 100 ml. 2 wk. after recontinuing the feeding of the same trace-mineralized salt. The trace-mineralized salt contained cuprous iodide and 3,5-diiodosalicylic acid averaging 0.0074% iodine.

[1] Technical Contribution No. 318, South Carolina Agricultural Experiment Station, Clemson.

A second experiment was conducted to see if trace-mineralized salt containing iodine in the form of potassium iodide would give similar results. The two salts used in this experiment had been marketed by the same company, but the potassium iodide-salt was an older salt. Six heifers averaging 5 mo. of age had been fed a concentrate mixture containing 1% trace-mineralized salt (3,5-diiodosalicylic acid) since shortly after birth. The PBI averaged 20.25 γ per 100 ml. of plasma. The trace-mineralized salt was replaced with plain salt, and seven to nine days later the PBI content of the plasma averaged 3.35 γ per 100 ml. After the nine days, three heifers (Group 1) were placed on the concentrate containing 1% trace-mineralized salt (3,5-diiodosalicylic acid). The PBI content averaged 9.97 γ per 100 ml. of plasma six days later, 12.52 at 12 days, and 16.65 at 16 days. At the same time, the three other heifers (Group 2) were fed trace-mineralized salt (KI). The PBI of these heifers' plasma averaged 3.85 γ per 100 ml. at six days, 4.96 at 12 days, and 3.56 at 16 days. The detailed data are presented in Table 2.

From the two experiments presented above, it appears that the feeding of trace-mineralized salt containing cuprous iodide and 3,5-diiodosalicylic acid can cause an elevation of PBI values in blood plasma. Trace-mineralized salt with potassium iodide as the source of iodine gave results similar to those obtained when all iodine supplement was removed.

Testing the trace-mineralized salt for iodine by the modified Brown method gave negative results. Generally, it is considered that compounds as simple as diiodotyrosine are measured by the technique. It is suggested that the 3,5-diiodosalicylic acid combines with some compound or compounds in the blood to give the high PBI values. These combinations disappear

TABLE 1

PBI values of heifers' plasma during periods when trace-mineralized salt and plain salt were fed (γ/100 ml.)

	Trace-mineralized salt fed[a]			Plain salt fed			Trace-mineralized salt fed[a,b]
Heifer	February 19	February 20	Av.	March 6	March 11	Av.	March 25
H976	15.70	16.22	15.96	4.20	3.65	3.93	11.46
H977	17.01	14.18	15.60	2.84	4.07	3.46	11.10
H978	21.31	19.24	20.28	4.60	3.31	3.96	12.03
H980	32.63	32.63	32.63	3.96	3.26	3.58	12.99
H981	24.54	21.85	23.20	4.08	4.14	4.11	5.75
G724	9.63	9.63	9.63	3.72	3.58	3.65	11.89
Av.	20.14	18.96	19.55	3.90	3.67	3.78	10.87

[a] Trace-mineralized salt contained cuprous iodide and 3,5-diiodosalicylic acid.
[b] Feeding began again on March 12.

TABLE 2

PBI values of heifers' plasma during periods of feeding trace-mineralized salt and plain salt (γ/100 ml.)

Heifer	Trace-mineralized salt fed[a]			Plain salt fed			Trace-mineralized salt fed[b]		
	May 22	May 23	Av.	May 30	June 1	Av.	June 6	June 11	June 15
				Group 1					
H992	22.88	19.91	21.40	3.05	4.30	3.68	11.56	13.54	16.79
X527	16.11	16.11	16.11	3.05	3.47	3.26	8.32	9.80	14.70
X528	24.86	24.86	24.86	3.79	3.76	3.78	10.02	14.23	18.46
Av.	21.28	20.29	20.79	3.30	3.84	3.57	9.97	12.52	16.65
				Group 2					
G732	13.63	14.50	14.07	1.96	2.70	2.33	3.51	4.55	3.29
H991	20.22	20.22	20.22	3.43	3.99	3.71	4.08	5.79	3.74
X529	24.88	24.88	24.88	2.81	3.86	3.34	3.96	4.55	3.65
Av.	19.58	19.87	19.72	2.73	3.52	3.13	3.85	4.96	3.56

[a] Trace-mineralized salt containing cuprous iodide and 3,5-diiodosalicylic acid fed to Groups 1 and 2 until May 23.

[b] Trace-mineralized salt feeding begun again on June 1, P.M. Group 1 received the cuprous iodide and diiodosalicyclic acid salt, and Group 2 received potassium iodide salt.

from the plasma within seven days, but it takes more than 16 days of feeding to return the PBI to as high levels as they were previously.

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Symposium on Cottage Cheese

RECENT DEVELOPMENTS IN COTTAGE CHEESE MANUFACTURING PROCEDURES

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Outstanding work of permanent benefit to the cottage cheese industry has been done—and we know is still under way—under the direction of many of our contemporaries, some of whom are present today. It is essential to salute their accomplishments which today are helping to make the growth of cottage cheese the most dramatic ever witnessed in the cheese industry. We should thank them all, but can name only a few: Elliker, Collins, Babel, Lundstedt, Harmon, Doan, Deane, Czulak, and their associates for their outstanding work on keeping quality and starters and their attributes; Ashworth, Coulter, McMeekin, Jenness, Herreid, and their coworkers for scientific knowledge of the proteins and properties of milk and dry milk; Reid, Zakariasen, Parmelee, Collins, Wilkowski, Hedrick, Tuckey, Henson, and their colleagues for information on milk composition, nonfat utilization, and yields; Lundstedt, Jensen, Stone, Tretsven, Phillips, Fouts, Angevine, Schock, Ramsey, Hales, and Honer for manufacturing ideas and techniques; and health officials in many states and in the U. S. Public Health Service for guidance in the protection of this perishable food product. The contributions of men such as these are permanently established in the literature and in industrial practices.

At this time you and your program committee expected us to discuss specifically some of the recent developments in cottage cheese manufacture with which our research team at Wisconsin has been concerned. The men on this team include A. M. Swanson and our former colleague, D. B. Emmons, who is now associated with the Dairy Technology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa. We propose to talk about developments in manufacturing concerned with some of our studies of cutting acidity, the influence of starters on acidity and firmness of coagulum and, finally, to add a comment about our interest in the Grade A cottage cheese program, a most significant regulatory development.

[1] Papers presented at the Cottage Cheese Symposium, 54th Annual Meeting of the American Dairy Science Association, University of Illinois, Urbana. 1959.

MILK FOR COTTAGE CHEESE

We start this discussion with the assumption that the milk and milk products we are using must be of low bacterial count and from healthy udders. We recognize that the influence of solids-not-fat (SNF) content of the milk is highly significant and, under some circumstances, that SNF must be increased or supplied initially by nonfat dry milk. While under certain conditions this permits certain economies and efficiencies in manufacture, we believe that these modifications can pose unexpected problems of biological or chemical nature, accidentally or otherwise, some of which we propose to discuss. Incidentally, we find a common misunderstanding of our federal definitions concerning the use of nonfat in making cottage cheese. While the nonfat may be of unreproachable quality, the water used and techniques of handling during reconstituting sometimes may be questioned. The provision requiring that reconstituted or fortified nonfat milk or cream dressing must be pasteurized before using for cottage cheese seems most reasonable.

CUTTING ACIDITY

The operation of cutting is vital to the efficiency of cottage cheese making because it affects: the percentage of solids recovered in the curd; the uniformity of curd particles; the composition, yield, and the quality of the cheese. If the acidity at cutting is too low, the cheese will be tough and rubbery in body and will leak whey in the package. If the acidity is too high, the curd particles will be broken, and the cheese will show gritty, hard particles or pastiness. It will be fragile and easily broken in cooking. It will be difficult to cream.

Cutting "by the clock" is usually the least desirable method of determining the proper time. It is usually accompanied by the comments: "I can't wait any longer!" or "I used a little extra starter so it would be ready on time."

Cutting by the split of the curd is not uncommon, sad to say. It can be exactly right with a no-rennet curd. If rennet action dominates the curd formation with low acidity, then the split test will produce the defects of low acidity mentioned above.

Cutting by acid development is the most critical method of fixing the time of this important step.

Three tests can be used in detecting the right acidity for cutting. They are: 1) titratable acidity of whey, 2) pH of curd, and 3) the Acid Coagulation (A-C) Test. All are excellent when properly used—yet all are subject to certain limitations.

The titratable acidity of whey should be made on clear whey, free of curd particles. This clear whey can be obtained by drawing curd into a pipette at a point 3 to 6 in. below the surface of the curd. This curd is vented into a Babcock cream test bottle and centrifuged for about 3 min. The clear whey can be poured out of the test bottle, leaving the curd behind.

Clear whey obtained from the top of the vat by any method will usually show the chosen acidity for cutting after the acidity has already exceeded the proper level for cutting in the whey centrifuged from the curd. Temperature, time of adding rennet, composition of milk, and strength of coagulator will all influence the separation of whey on top of a vat of cottage cheese. Once this whey has separated from the curd, its acid development is greatly delayed by the lack of lactic acid–producing organisms in it.

The correct titratable acidity for cutting depends on such factors as milk composition, the previous heat treatment of the milk, and the starter used in making.

The pH of curd when used to determine the time of cutting should be made on a sample taken from 3 to 6 in. below the surface of the vat.

The pH can be measured with a glass electrode or the quinhydrone electrode, provided the apparatus is used correctly and is checked against a fresh buffer of known strength.

The A-C Test (Emmons *et al.*, Wisconsin Extension Circular 541. 1957) offers a simple method for determining the time of cutting the curd for greatest efficiency in manufacturing. This test is simply a method of detecting when milk coagulates by starter action alone. The skim, which contains the starter but no rennet coagulator, is held at vat temperature. The end point of this test is indicated by the first appearance of whey in cuts made with a spatula in the first detectable soft gel.

It follows, of course, that acid production must proceed at the same rate in the test sample and the vat, and the rate of acid development must be the same throughout the A-C test sample.

Standards for titratable acidity of the clear whey and pH measurements of the curd can be set by this test if it is applied in the laboratory to the identical milk and starter to be used in making the cheese in the vat. Thus, the test can be used directly by the cheesemaker, or it can be used by the laboratory technician whose responsibility it is to determine the correct cutting acidity and to give the maker specific instructions in terms of titration of the whey or pH of the curd.

The application of the A-C test in selecting the optimum cutting acidity is shown by the data of Tables 1 and 2.

TABLE 1

Effect of total solids in skim on the acidity when the A-C end point is used to determine the time of cutting

Acidity equivalents to A-C Test at cutting:		
Total solids in milk	Titratable acidity of whey	pH of curd[a]
(%)		
8.7	0.50	4.59
9.6	0.55	4.61
10.5	0.62	4.60
12.4	0.74	4.56
14.3	0.86	4.55

[a] Determined on curd from which a portion of the whey had been removed.

TABLE 2

Effects of heating skim on acidity at the cutting time of cottage cheese curd

Acidity equivalents to A-C Test at cutting:		
Heat treatment	Titratable acidity of whey	pH of curd[b]
	(%)	
145 for 30 min.	0.50	4.61
155 for 30 min.	0.49	4.63
165 for 30 min.	0.47	4.68
175 for 30 min.	0.33	5.09
High-heat NDM[a]	0.51	4.93

[a] Reconstituted to 11.1% T.S.; other lots 8.5% T.S.

[b] Determined on curd from which a portion of the whey had been removed.

Table 1 shows the trend of effects of total solids in skim on the correct acidity of cutting curd for cottage cheese. Titratable acidity required for proper working of the curd increases as the total solids increases; the pH of the curd at cutting decreases with increasing total solids. These cutting acidities were determined by the A-C Test and indicate, we believe, the trends of changes in any normal operation.

The pH at which the A-C end point occurs decreases as the total solids in the milk is increased. This effect was studied by adding chlorides or phosphates of sodium and calcium, or a mixture of simulated milk salts to skim-milk to increase the ionic strength 0.10 at pH 4.7. These additions all decreased the pH at the A-C end point, but in varying degrees de-

pending upon the salts used. It appears to us that much of the decrease in pH at the A-C end point of skimmilks which are reconstituted with increasing amounts of total solids may be explained by the increased ionic strength of milk salts in solution and their effect upon the pH range of minimum solubility of casein.

Table 2 shows the tendency for titratable acidity in whey to decrease and for pH to increase at the A-C end point with increased heat treatments of the skimmilk. Again, these data are to show the trend of the effects rather than to serve as standards for any single manufacturing operation.

The titratable acidities and pH values cited in Tables 1 and 2 include variations not commonly found in any single plant's operation. For each treatment illustrated those acidities which were apparent at the A-C end point were preferred for optimum working qualities of the curd. The A-C Test has been particularly useful in making cottage cheese from skimmilk fortified or reconstituted from nonfat with an unknown history of previous heat treatments. A year ago we reported the successful manufacture of cottage cheese from skimmilk heated as high as 175° F. for 30 min. This was made possible largely by knowing how to use the A-C test to detect the optimum acidity for cutting and by using increased amounts of rennet.

EFFECTS OF LACTIC CULTURES ON ACIDITY AND FIRMNESS OF CURD

It is a well-known fact that cultures do not always show the same firmness of curd. This is important when they are used as starters for making cottage cheese. A total of 17 commercial cultures were compared in a series of trials. Four of them were obtained from Heinemann (1), who has also been interested in this phenomenon. All cultures were grown in the same lot of nonfat dry milk. Heinemann (2) suggested that different proteolytic activity caused different curd strength of cottage cheese curd at any given acidity.

It seemed possible that other factors also might be involved in determining the relation between cultures and curd firmness.

Measurements of titratable acidity, pH, and curd strength were made at intervals over a rather wide range of acidity and curd strength after the initial coagulation of the cottage cheese curd.

The relationship of curd strength to titratable acidity of whey was not very close (coefficient of correlation was .59). Cultures varied widely; some showed high curd strength at 0.55% titratable acid in the whey, while others did not even coagulate the skimmilk until the titratable acidity had reached or exceeded 0.55%.

An interesting fact was the observation that increases in acidity (decreases in pH) after coagulation gave similar increases in curd strength ($r = .97$); all cultures were alike in this respect.

Titratable acidity of whey showed some of the same relationships to curd strength as did pH measurements of the curd. Even though the titratable acidity of the whey at coagulation of the cottage cheese curd varied from 0.38 to 0.58%, still the increase in acidity after coagulation was quite closely related to the strength of curd developed ($r = .83$). Cultures, though, were important in determining the rate of increase in curd strength for given increases in acidity.

Despite these similarities, in some respects the cultures showed distinctly different relationships between the titratable acidity and pH measurements made during the formation of the cottage cheese coagulum. Differences were obviously associated with the different cultures used.

Different cultures showed different titratable acidities at the same pH. At pH 5.1 the titratable whey acid was 0.66% for Culture G, and for Culture P, 0.44%. At a titratable acidity of 0.55% in the whey the pH varied from 5.25 for Culture G to 4.79 for Culture P.

Cultures which developed a relatively high titratable acid at the relatively constant pH at the A-C end point produced floating curd.

Activity of cultures can be measured by the Horrall-Elliker test. But starters may have good activity while reaching 0.4% titratable acid at 100° F., and low activity in the zone immediately preceding milk coagulation. For example: Cultures with activities of 0.48, 0.45, and 0.52% required 7.3, 4.4, and 4.8 hr. at 90° F., respectively, to reach the A-C end point at 4.75 pH. Previous heat treatment of the milk, or differences in temperature of incubation between the activity test and the cheese vat, could account for these differences in starter effectiveness.

TABLE 3

Titratable acidities of whey at the A-C endpoint for different cultures and the tendencies of curd to float at the end of the heating process

Action	No. of cultures	No. of trials	Range of acidity of whey at the A-C endpoint
			%
Sinking	8	19	0.57–0.64
Floating	5	12	0.67–0.79

Explanation of starter effects. Variations in titratable acidity of different cultures at the same pH can be explained by the probability that the higher titration was undoubtedly caused by the presence of carbon dioxide, acetic acid, or other weak acids in the end-products of the fermentation. Carbon dioxide is largely undissociated at pH 5.0 and below. Thus, the production of this weakly ionized acid by a culture

would not lower the pH appreciably, yet would contribute significantly to the titratable acidity test. The variations make it clear that errors possible in using titratable acidity rather than the A-C Test to select the proper time of cutting can produce defects in curd making directly attributable to either too high or too low pH. With unusually high titratable acid at the normal pH of cutting, the faults of floating curd might be expected.

The results of this study may also explain those occasional baffling vats of skimmilk for cottage cheese that do not coagulate even though the titratable acidity is right. Such vats should be checked for pH which, at the time of cutting normal skimmilk at the A-C end point, will approximate 4.7.

Incidentally, the starters which are usually selected for their high aroma when used for buttermilk may exhibit abnormally high titratable acid at the time of coagulation. Such starters, according to our observations, could actually be responsible for floating curd if used for cottage cheese making.

SIGNIFICANCE OF GRADE A COTTAGE CHEESE

When the State of Indiana recently recommended the techniques and standards necessary for the production of Grade A cottage cheese, a chain reaction was started. Thinking consumers approve such ideals. Competitors must inevitably match such standards. The influence of the regulations of the State of Indiana is being felt in ever-widening areas. Regulations now developing in such areas indicate the trend the cottage cheese industry is taking today with the help of the U. S. Public Health Service. The 1953 Milk Ordinance and Code recommended by the Public Health Service included cottage cheese as a milk product, and the applicable requirements of the Ordinance and Code would hold. But there is a provision for states and communities to exempt cottage cheese.

The principles involved in the manufacture of Grade A cottage cheese can be applied profitably wherever the highest quality of product is needed. Public health officials recognize herds as a source of contamination of milk which, though serious, can be controlled by known methods of protection and pasteurization. Men and women involved in production or handling of cottage cheese, like any food handlers, are potential sources of contamination to be controlled. Finally, the weaknesses of the open vats and other equipment associated with the manufacture of cottage cheese must be eliminated to achieve the utmost in positive control of sanitary production.

Trends of regulations developing for the control of production of Grade A cottage cheese follow closely those recommended for the handling of Grade A milk. Milk will satisfy Grade A regulations, as will the construction and maintenance of the plant in which the product is manufactured. Space requirements will be dictated by the paramount consideration of the protection of the public health. Water, which is such an important factor in cottage cheese production, will be pasteurized or chlorinated and handled in sound pipes and filters.

Methods of production in meeting these regulations present problems which will be solved gradually. Minimizing contamination of persons, some operators believe, will affect not only choice of working clothes, but may even require the use of face masks. Hand-washing facilities convenient to the operations are essential. Hand filling of cartons—from the vat or elsewhere—will disappear in favor of covered mechanical operations to take care of filling and capping. Coliform and mold and yeast counts of less than ten per gram may be set at lower levels eventually as methods and equipment can be improved.

The significance of such standards in the mechanics of producing cottage cheese are obvious. Under the guiding influence of such ideals, there must be developed eventually a completely covered system of manufacturing and handling curd in vats, conveyors, mixers, packagers, and cappers. More adequate refrigeration facilities and techniques will be needed to reduce temperatures of the product quickly to less than 40° F., and to maintain them uniformly. These are some of the characteristics of the best practices which will prevail when production and distribution of Grade A cottage cheese becomes an accepted and well-established procedure.

The economic significance of such developments is not hard to imagine. Investments in improved equipment and labor-saving devices will tend to limit the number, and increase the size of plants producing Grade A cottage cheese. We should expect improvements in quality and shelf-life of the cheese manufactured under such conditions. The status of an inefficient cheese operation can not long remain in doubt if it fails to meet the qualifications of a Grade A product.

Finally, to the statesmanship of the American Cottage Cheese Institute, and to all who are directly responsible for the production and distribution of cottage cheese, these standards and requirements present a challenge today to meet the needs of tomorrow.

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COTTAGE CHEESE CULTURES [1]

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The addition to pasteurized skimmilk of a bacterial culture capable of producing lactic acid from lactose is the first step in the process of cottage cheese manufacture. The function of this culture is to produce sufficient lactic acid to coagulate the casein. Although various coagulators containing the enzyme rennin frequently are used in the manufacture of cottage cheese, it is essential that the skimmilk undergo acid coagulation rather than rennin coagulation. For the successful manufacture of cottage cheese, it is also important that the skimmilk undergo acid coagulation in a quiescent state; therefore, the addition of lactic acid to the milk is not a satisfactory substitute for acid production by a bacterial culture.

BACTERIAL FLORA OF COTTAGE CHEESE CULTURES

The most common culture employed in cottage cheese manufacture consists of a mixture of lactic acid–producing and citric acid–fermenting organisms. The acid-producing organisms are either *Streptococcus lactis* or *Streptococcus cremoris* and the citric acid–fermenting species are either *Streptococcus citrovorus (Leuconostoc citrovorum)* or *Streptococcus paracitrovorus (Leuconostoc dextranicum)*. However, cottage cheese has been manufactured successfully with pure cultures of either *S. lactis* or *S. cremoris*. Both types of cultures have certain advantages and limitations.

Cottage cheese manufactured with a culture containing both lactic acid–producing and citric acid–fermenting bacteria generally has more flavor, due to biacetyl and volatile acids, than cottage cheese manufactured with acid-producing organisms only. This is particularly true of cottage cheese manufactured by the long-set method and cut at a high acidity. Skimmilk set at 70 to 72° F. with a culture capable of producing considerable biacetyl will contain as much as 2.6 p.p.m. biacetyl at the time of cutting (pH 4.7). Much of the biacetyl contained in the coagulum at the time of cutting is removed with the whey, but a culture producing large amounts of biacetyl yields curd with a correspondingly higher biacetyl content (2).

Certain citric acid–fermenting organisms also have an influence on the rate of acid development by the lactic acid bacteria. Such an effect is shown by the data presented in Table 1. The *S. lactis* and *S. citrovorus* cultures used in this trial grew well in combination and produced a very satisfactory flavor. No lactic acid was formed in milk by the *S. citrovorus* culture, so the increase in acidity by the combined cultures is due principally to the stimulation of *S. lactis*. At relatively high acidity, *S. citrovorus* does produce appreciable amounts of acetic and propionic acid and some of the titration value late in the incubation period may be attributed to these acids. However, the stimulating effect on *S. lactis* is evident early in the incubation period and at a low titratable acidity.

TABLE 1

Rate of acid production in skimmilk inoculated with 1% *Streptococcus lactis* and/or *Streptococcus citrovorus* and incubated at 86° F.

Test culture(s)	Per cent titratable acidity after incubation for:				
	0 hr.	2 hr.	4 hr.	6 hr.	8 hr.
S. lactis	0.19	0.19	0.23	0.36	0.64
S. lactis + *S. citrovorus*	0.20	0.21	0.35	0.66	0.82
S. citrovorus	0.19	0.19	0.19	0.19	0.19

Some cottage cheese manufacturers object to the use of cultures containing *S. citrovorus* or *S. paracitrovorus*, on the basis that they produce more carbon dioxide than pure cultures of *S. lactis* or *S. cremoris*. Floating curd and spongy curd have been attributed to the activity of these citric acid–fermenting bacteria. The large amounts of cottage cheese manufactured with cultures containing both types of organisms, without evidence of excessive gas, indicate that these cultures generally give satisfactory results. Occasionally, a culture is examined which contains a much larger percentage of *S. citrovorus* or *S. paracitrovorus* than most cultures. However, such a condition is unusual and may be related to constant overripening of the culture.

Cottage cheese manufacturers who prefer to use pure cultures of *S. lactis* or *S. cremoris* indicate that such cultures are more uniform in acid production from day to day, they produce very little carbon dioxide, and testing procedures for bacteriophage are simplified. The bland flavor of the cottage cheese manufactured with such cultures is not considered objectionable by those who advocate their use.

Cultures sold commercially may contain organisms other than *S. lactis, S. cremoris, S. citrovorus,* or *S. paracitrovorus. Streptococcus diacetilactis,* an organism having the ability to produce lactic acid from lactose, and acetylmethylcarbinol, biacetyl, volatile acids, and carbon dioxide from citric acid, is the predominant organism in some cultures; particularly

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cultures imported to this country from Europe. Cultures containing this organism can not be distinguished from those containing the usual types of organisms by organoleptic or microscopic observations. Chemical analyses for fermentation products can be used to distinguish *S. diacetilactis* from *S. lactis* or *S. cremoris*. The amounts of lactic acid, acetylmethylcarbinol, and biacetyl produced by a culture of *S. diacetilactis* growing in skimmilk are shown in Table 2.

TABLE 2

Amounts of lactic acid, acetylmethylcarbinol, and biacetyl formed in skimmilk by *Streptococcus diacetilactis*

Compound	Amount of compound produced in skimmilk by a 1% inoculation
Lactic Acid	0.36% produced in 6 hr. at 86° F.
Acetylmethylcarbinol	32.2 p.p.m. produced in 16 hr. at 70° F.
	108.4 p.p.m. produced in 16 hr. at 70° F. when 0.20% citric acid was added to the skimmilk.
Biacetyl	1.6 p.p.m. produced in 16 hr. at 70° F.
	14.7 p.p.m. produced in 16 hr. at 70° F. when 0.20% citric acid was added to the skimmilk.

The analyses indicate that *S. diacetilactis* produces lactic acid rapidly at 86° F., it forms both acetylmethylcarbinol and biacetyl in milk, and the amounts of carbinol and biacetyl produced by the organism can be increased by the addition of citric acid to milk. Sandine *et al.* (3) found that the *S. diacetilactis* cultures isolated by them could be divided into two groups: (1) Those forming large amounts of both acid and gas, and (2) those forming small amounts of acid and considerable gas. Both groups were considered capable of causing floating curd during cottage cheese manufacture.

FACILITIES FOR CULTURE PROPAGATION

Within the past few years, a number of manufacturing plants have provided more suitable facilities for culture propagation. There are several reasons for the construction of such facilities. (1) Production in many plants has increased due to consolidations or other factors and, therefore, the extent of loss incurred with a culture failure is proportionately greater. Facilities are being provided to guard against excessive product loss. (2) More personnel who have a good knowledge of the problems associated with cultures are being employed by the dairy industry. These people know what facilities are required for satisfactory culture propagation and are demanding such facilities. (3) When a plant constructs new facilities and, thereby, eliminates culture problems that were quite prevalent previous to such construction, surrounding plants try to attain the same result with similar facilities.

Some of the newer culture rooms provide an area within the plant that is accessible only from the outside, or where there is little chance of contamination from manufacturing operations. The area is sealed off from the rest of the plant and is equipped with a special ventilating system. One of the main functions of the ventilating system is to provide a slight pressure within the culture rooms so that contaminated air will not enter. Air entering the culture area is usually brought in from an outside source and passed through special filters, or treated in some way to prevent possible contamination of cultures. Special culture facilities have eliminated many culture failures when properly constructed and where satisfactory procedures for culture propagation are in effect. Areas designed for propagation of mother cultures, and preparation of both intermediate and bulk culture, are the most satisfactory. Some areas have been constructed properly, but sufficient care has not been taken to prevent contamination. Cultures which contain bacteriophage, or entrance by personnel engaged in cheese manufacture, are the most common sources of contamination of these areas.

RECENT ADVANCES IN CULTURE TECHNIQUES

A common procedure in cheese plants is to obtain a culture from a supplier and propagate the culture from day to day as a mother culture. The mother culture is used to prepare an intermediate culture, and the intermediate culture is used to prepare bulk culture employed in cheese manufacture. Therefore, three propagations are made before the culture enters the cheese vat. The addition of bulk culture to the cheese vat, plus the usual incubation, can be considered a fourth propagation. Therefore, slight contamination within the mother culture may lead to excessive contamination in the cheese vat.

There are lyophilized cultures on the market today which contain a sufficient number of viable organisms to produce about 3 gal. of culture in 14 or 16 hr., using incubation temperatures of 70 to 72° F. It is possible, therefore, to dispense with propagation of mother cultures and use commercial lyophilized cultures for preparation of the intermediate culture. This procedure has been used by a number of plants for several years with considerable success. The method eliminates one propagation of the culture and this is particularly advantageous in plants experiencing slow acid development due to bacteriophage. Another advantage of this procedure is that one lactic acid–producing strain within the culture does not become dominant with repeated transfer. Certain commercial cultures contain several strains of lactic acid–producing bacteria, with

different bacteriophage sensitivities, and a proportionate amount of each strain is mixed together just prior to drying. If the culture is propagated for a long time, one strain may become dominant and the culture no longer has some of the advantages of a multiple-strain culture. A single bacteriophage strain can cause complete cessation of acid production in a culture where one strain becomes dominant, whereas this would not be the case if the culture had the same original bacterial composition.

Intermediate culture is being prepared from frozen culture in some cheese plants. This procedure also eliminates daily culture propagation. A rather large batch of culture is prepared and frozen in paper milk cartons. After freezing, the product is tested for activity in the laboratory and in actual cheese-making operations. If satisfactory results are obtained from these tests, the plant may draw from the frozen supply for several weeks. The method has some of the same advantages as preparation of intermediate culture from a lyophilized culture. However, the opportunity for contamination is generally considered to be greater in a commercial plant than in a culture laboratory.

Cultures in manufacturing plants not only become contaminated with yeast, mold, and bacteriophage, but they also become contaminated with strains of other lactic acid–producing streptococci. On several occasions, cheese plants having culture failures due to bacteriophage have sent samples of their culture to our laboratory and indicated the original source and culture number. Examination of the cultures showed that a contaminating strain had become dominant and changed the phage sensitivity pattern of the culture. The ease with which cultures can be contaminated in a cheese plant with lactic acid–producing streptococci has been revealed by some plants carrying cultures of *Propionibacterium shermanii, S. citrovorus,* etc. Therefore, there are several reasons for not propagating cultures for excessively long periods of time without renewing them.

Special media for culture propagation are now available to the dairy industry. Nonfat dry milk solids can be purchased in sealed packages sufficient to prepare about two-thirds of a quart of reconstituted milk for propagation of mother cultures. The powder is pretested to make certain that it supports active growth of lactic cultures. Larger quantities of pretested powder can be obtained for the preparation of intermediate or bulk culture. Sterile skimmilk in sealed cans also is being offered for mother culture propagation. This product simplifies culture propagation because it eliminates the necessity for heat treatment and subsequent cooling of the culture medium. The sterile skimmilk is pretested to insure satisfactory culture growth. Nonfat dry milk solids with a low calcium content is available in some areas as a culture medium. When reconstituted with water that is low in calcium, the medium prevents phage multiplication. However, reconstituted milks prepared with some lots of the low-calcium powder do not support the growth of lactic cultures as well as does regular skimmilk (1).

Special vessels have been introduced for the preparation of mother, intermediate, and bulk culture. All are designed to prevent the entrance of contaminating microorganisms or bacteriophage to the culture medium subsequent to heating. After milk is heated, the decrease in volume due to cooling results in air being drawn into a culture vessel. The special vessels have facilities for filtering the incoming air. In addition, the vessels permit inoculation with little chance of contamination.

SUMMARY

The cottage cheese manufacturers in the United States have a wide variety of cultures at their disposal. Most of these cultures have greater initial activity than those manufactured several years ago, and there are greater differences in their bacteriophage sensitivity patterns.

The personnel associated with cottage cheese manufacture recognize that the lactic culture is the most important single factor contributing to a successful operation. Therefore, they are attempting, by various means, to maintain the activity and purity of their cultures. The fact that the same culture, from the same supplier, works well in one plant and not in another clearly indicates that the basic culture is satisfactory but that the extent of contamination and quality of milk vary among plants.

Pretested media for culture propagation eliminate one possible source of culture failure. The trend toward more frequent renewal of cultures and fewer propagations of a culture before being used in manufacturing has considerable merit.

Protecting cultures from air contamination by providing a culture-manufacturing area and the use of specially designed culture vessels should contribute much toward a sound culture program.

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FACTORS AFFECTING THE YIELD OF COTTAGE CHEESE

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The major interest of plant operators in cottage cheese yields has centered around those factors affecting the amount of curd that can be recovered from a definite amount of skimmilk. Curd yields are important to the industry because they affect profits, and cottage cheese is one of the more profitable dairy products. If we may assume a total raw material and processing cost of 12¢ per pound of dry curd with a yield of 15 lb. of curd per 100 lb. of skimmilk of 9% solids (1.67 lb. curd per pound of solids), an improvement in that yield of 0.5 to 15.5 lb. per hundred or 1.72 lb. curd per pound of solids would mean a reduction of 0.4¢ per pound in the cost of curd. This is a very significant figure, when any possible reduction in costs without sacrifice of quality is important. It is believed that an increase in cottage cheese curd yields to the extent indicated above is attainable in many operations.

WHAT IS GOOD CURD YIELD AND HOW SHOULD IT BE CALCULATED?

Several investigators have called attention to the fact that it is the protein content of the milk, principally the casein, that is the determining factor in yield insofar as the composition of the milk is concerned. It is true that an accurate expression of curd yields should take into account the casein content of the skimmilk as well as another factor of limiting importance; namely, the moisture content of the curd. Such accuracy is important if we are to determine the efficiency of a plant in terms of its available milk supply and its success in recovering a satisfactory proportion of the solids in that supply. Such accuracy is also important if we are to make meaningful comparisons between various plants in terms of the recovery of a satisfactory proportion of solids. For practical purposes, however, the actual pounds of curd secured in the daily operation under routine operating conditions, curd of such a moisture content that will produce a satisfactory finished product and one that will comply with the legal standard for moisture, is the important figure and, when converted to pounds of curd per pound of solids, is a practical measure of plant efficiency. An important factor in determining that efficiency is the closeness of control over the moisture content of the curd and of the finished product.

Commonly used methods for the expression of yields on a practical basis are (1) pounds of curd per 100 lb. of skimmilk, and (2) pounds of curd per pound of solids in the skimmilk. It is believed there would be common agreement that a curd yield of 15.5 lb. per 100 lb. of skimmilk of 9% solids, or 1.72 lb. of curd per pound of solids, is a good, satisfactory yield with present conventional methods of making cottage cheese. Some plants improve this yield by better recovery of solids from skimmilk fortified with added solids-not-fat in the form of low-heat condensed skimmilk or spray process nonfat dry milk, and for such operations it is believed that a yearly average of 1.80 lb. of curd per pound of solids is a workable, attainable plant standard, based on the actual pounds of curd taken from the vat. Fluctuations below this figure in the summer and above it in the cold winter months are commonly experienced, probably due to variation in the solids content of the milk.

A third basis of expressing curd yield is the pounds of curd per pound of casein, and since casein is by far the most important cheese-making constituent of the solids, it is perhaps the most accurate of the indices commonly used. Assuming a fortified skimmilk of 11.0% solids with a casein content of 3.2%, our standard yield of 1.80 lb. of curd per pound of solids becomes 6.18 lb. of curd per pound of casein. Lundstedt (6) calculated that the correct factor to use in estimating the pounds of curd of 79% moisture per 100 lb. skimmilk of 9.0% solids was six times the casein content, when 36.2% of the solids is retained in the curd and the casein is 28.7% of the skimmilk solids. On this basis, the casein content of the skimmilk was 2.58% which, divided into the yield obtained of 15.5 lb. of curd, gave the factor of 6.

The Walker casein test, employing formalin to free the acidic protein groups for titration against 0.1 *N* sodium hydroxide, may be used to secure quite accurate figures on the casein content of the fluid or fortified skimmilk in the cheese vat. It should be in common use in cottage cheese plants and has special value at times when yields are running abnormally low or high.

FACTORS AFFECTING CURD YIELDS

Curd yields are determined by the interaction of a number of factors and the accuracy of the final yield figure is dependent upon the accuracy with which these various factors are determined. The following items must be taken into consideration:

1. *The composition of the skimmilk with particular reference to the total solids and protein content.* Factors influencing the total solids and protein content of whole milk and the skimmilk separated from it are (1) breed of the cow, (2) stage of lactation, (3) season of the year, and (4) diseased conditions of the udder, such as mastitis. In general, total solids varies with the

fat content and higher curd yields are secured from skimmilk separated from high-fat milk, as reported by a number of investigators and confirmed in plant practice. The high fat, protein, and total solids content of Jersey and Guernsey milk as compared to Holstein milk has been demonstrated by Overman and associates (8), and Turner (10) has prepared a table from their data which has been quite widely quoted. Jacobsen and Wallis (5), in their study of factors that affect the composition of milk, found a decrease in total solids content during the first 3 mo. of lactation with the Jersey, Guernsey, Ayrshire, and Holstein breeds. A general trend upward was noted during the middle months and the last 4 mo.

Seasonal variations in the composition of milk have been studied by a number of investigators. Honer and Herzer (4), reporting on the composition of Mississippi milk, stated that the casein content followed and appeared largely responsible for the over-all fluctuation in the total protein content. The highest average for casein appeared during November at 2.89% and the lowest during February at 2.55%. Declining gradually from November through March, the average casein trend remained low from March through July, then steadily increased to November. An extensive two-year study of the geographic and seasonal variations in the composition and vitamin content of nonfat dry milk solids was reported by O'Malley, Baldi, and Gross (7) in 1944. Their results showed that the protein content was lowest in February, March and April, with the lowest average in March (35.98% in the powder), and that it was highest in September, October, and November, with the highest average in October (38.21%). White and Judkins, as reported by Jacobsen and Wallis (5), found that regardless of the time of calving, the percentages of fat and of solids-not-fat were lower during the warm months and higher during the cold months of the year. It is readily apparent that when the seasonal effects contributing to low solids and low protein content are superimposed on similar effects for the period of lactation, abnormally low yields are the result.

Milk from cows suffering from mastitis is noted for its poor cheese-making qualities, because of its lowered casein content, increased content of whey proteins, higher pH, and other abnormalities. Undoubtedly, the inclusion of such milk in mixed supplies has reduced curd yields in cottage cheese plants and produced abnormal conditions of coagulation in the vats. Waite and Blackburn (11) recently reported on the chemical composition and the cell count of milk and stated that when the cell count approached 1,000,000 per milliliter, the casein content of the milk was decreased; with a 500,000 cell count, there was decreased production and less solids-not-fat content. A condition of subclinical mastitis led to a decreased solids-not-fat content.

In any event, whether the solids content of the skimmilk is low, high, or average, we must know what it is, if we are to get an accurate expression of curd yields on the preferred basis of pounds of curd per pound of solids. Using the Mojonnier solids test is the most accurate method, but a lactometer may be used with a table for converting lactometer readings into per cent solids. Supplies of fluid skimmilk should be tested at least weekly throughout the year by the Mojonnier method, to obtain the trend of the solids content and permit greater accuracy in charging solids to the cheese-making operation.

FORTIFICATION WITH ADDED SOLIDS

The addition of low-heat condensed skimmilk or Extra-grade spray process nonfat dry milk to fluid skimmilk has been demonstrated to improve the recovery of milk solids in the cottage cheese curd. Bender and Tuckey (1), studying the recovery of skimmilk solids in cottage cheese curd, reported that 38.8% of the solids was recovered from skimmilk of 9.71% solids content as compared to 32.2% recovery for skimmilk of 8.92% solids. Theoretically, it should be possible to secure increasing yields with solids levels in the milk up to 15.0% or possibly higher, but handling very large amounts of curd under commercial conditions in conventional cheese vats becomes a difficult problem and the tendency has been to secure maximum yields per pound of solids at somewhat lower levels. It is believed that not much is gained, and some loss may be experienced, by fortifying skimmilk to more than 11.0% solids.

2. *Accuracy of determination of volume or weight of skimmilk.* In skimmilk reconstituted 100% from powder, the problem is simplified by the use of weighed bags. Weigh tanks, or storage tanks equipped with volumetric gauges, may be used for fluid skimmilk. In their absence, cheese vats may be calibrated by the use of milk or water meters with a marked stainless steel gauge in a fixed position in the vat, using water at the setting temperature. Repetitive filling of the vat with fixed, measured volumes of water is a laborious but sometimes necessary procedure. Hydraulic load cells mounted under the storage tank or under the cheese vat are under study at the present time and if found practical and accurate will be one answer to the problem of getting an accurate figure on the weight of skimmilk made into cheese.

3. *Accuracy of weight of curd or finished, creamed cheese.* Obviously, the most accurate figure on the weight of the curd is obtained by weighing it before creaming. This may be done in operations involving the use of mechanical mixers and, with careful control of the weight or volume of cream used, provides efficient composition control. In some small operations the curd is weighed into curd cans containing weighed or measured volumes of the creaming mixture. Here again, hydraulic load cells in-

stalled under the cheese vat would be the ideal means of obtaining the weight of the curd and would be useful in operations where creaming is done in the vat. In such operations now, an estimate is made of the weight of curd in the vat based on previous experience, and the creaming mixture added accordingly. To obtain a final yield figure, the amount of curd must be estimated from the final weight of the creamed, packaged cottage cheese, deducting the weight of the cream and the salt. This method involves a number of possible errors in the finished package count, over-weight in the cups, spillage, and loss in the packaging operation. An overweight of 0.4 oz. in 12-oz. cups reduces the estimated curd yield by 0.05 lb. per pound of solids.

4. *Moisture content of the curd.* The finished, packaged product, creamed or dry, must comply with the standard of not to exceed 80.0% moisture, which is the federal standard for interstate commerce and is commonly accepted to apply to intrastate business, as well. An efficient plant aims for control at the 79.0–80.0% level and sacrifices yield if the moisture falls below 79.0% (solids above 21.0%).

5. *Processing procedures as they affect loss of curd.* A number of details in the processing procedures may affect the recovery of solids in the curd or losses in the whey and wash waters.

(a) *Pasteurizing temperature and holding time for fluid skimmilk.* Probably most of the skimmilk manufactured into cottage cheese today is pasteurized by the high-temperature, short-time (HTST) method, and the usual commercial practice is to observe a minimum exposure of 162–163° F. for 16 sec., just above the diversion temperature of 161° F. High temperatures and long holding times may result in slow coagulation and a fragile curd.

Experimental work is being done with higher temperatures, thereby denaturing the whey proteins and including them with the casein during coagulation. Roberts (9) and associates reported that their best curd yields resulted when they standardized fresh skimmilk to 10–11% solids with low heat powder, pasteurized at 157° F. for 30 min., and added 0.068% calcium chloride before setting. Yields obtained were above 2.0 lb. of curd at 79% moisture per pound of solids. The use of quantities of calcium chloride greatly exceeding the legal maximum of 0.02% of the weight of the skimmilk seems to be an important part of the process. In view of the wide commercial application of HTST pasteurization, it is hoped that similar experimental work will be done with this method.

(b) *Solids-not-fat level in skimmilk.* Fortification of fresh skimmilk to 11.0% solids, or reconstituting powder to that level, has been found to increase curd yields and to give better yields than do higher solids levels. Occasional reports in the literature seem to confirm this conclusion.

(c) *Acidity at cutting time and proper relation of rennet and acid in coagulation.* Just enough rennet should be used to give the desired firmness of curd at the time the acidity has developed to the proper point for cutting. There are still differences of opinion as to the merits of determining optimum cutting points with pH or the acidity test. If acidity is used, rennet type of cheese made from 11% solids skimmilk should be cut at a whey acidity of about 0.56%. Most cheese makers using a pH meter will cut such curd at about 4.7. Small-curd cheese, cut with a ¼-in. knife, is commonly set with less rennet and cut at acidities of 0.02–0.04% higher than when the large-curd cheese is made.

(d) *The starter used.* While no definite and consistent differences have been noted between starters in different commercial laboratories, occasionally some starters give better yields of curd than others. Collins (2), in working with single-strain cultures of *S. cremoris* or *S. lactis* (no Leuconostoc species included), reported that these cultures yielded an average of 1.3% more cottage cheese than the mixed-strain cultures containing the flavor organism. This increase amounts to approximately 0.02 lb. additional curd per pound of solids. Preliminary work which we have done in a commercial plant gives a general confirmation of this tendency toward higher yields with cultures containing only the acid-producing organism.

(e) *Care in cutting.* Two men should be used in the cutting operation, one for each knife. A better job of cutting is done and excess shattering of curd can be avoided.

(f) *Stirring curd and cooking.* It is necessary that the initial stirring be done very carefully to avoid breaking the curd. Too many operators use vigorous stirring and cook too rapidly. It is better to withhold mechanical agitators until a temperature of 100° F. is reached in cooking.

(g) *Care in washing.* Operators sometimes become careless in washing the curd and break it in the process. There is some loss of curd in each wash-water and three rinses should be sufficient to wash the curd well and bring the temperature down to 40 to 43° F.

6. *Type of cheese made—large-curd or small-curd.* Some variation, of necessity, exists between companies and between cheesemakers with reference to the size of curd preferred and the size of knives needed. Commonly, a ⅝-in. knife is used for large-curd and a ¼-in. knife for small-curd cheese. Two plants making both types of curd, for which yield figures were obtainable, showed averages of 0.08 and 0.12 lb. curd per pound of solids more for large-curd cheese. A report on cheese yields in a Michigan plant recorded an average of 1.79 lb. of fine-curd cottage cheese per pound of powder, as compared to an average yield of 2.15 lb. for large-curd cheese.

In some instances, small-curd cheese is made without rennet. Bender and Tuckey (1) studied yields of cottage cheese curd secured with rennet coagulation, as compared to acid coagula-

tion, and reported that there was no difference in recovery of solids in the cheese between the two methods when proper manufacturing practices were followed. While not specifically stated, it appears that the same size of knife, namely 5/8-in., was used for cutting both types of curd.

7. *Fat content of creaming mixture.* This factor exerts its effect on the yield of finished creamed cheese obtained from a definite amount of curd. Regardless of the test of the creaming mixture, the finished product must contain not less than 4.0% fat, and if a mixture of a low-fat content is used, more of it is required to obtain the desired amount of fat. Such low-fat creaming mixtures have higher moisture contents and, therefore, the curd must be of lower moisture content than when high-fat creaming mixtures are used. The low-fat creaming mixture increases the amount of finished cheese obtained from the curd and decreases slightly the cost of materials per pound of cheese.

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KEEPING QUALITY OF COTTAGE CHEESE

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The great increase in cottage cheese consumption can be maintained by more uniform quality and extension of shelf-life. Some manufacturers do not realize that although cottage cheese is an excellent food for people, it is a very perishable product and also provides an excellent medium for the growth of many microorganisms. Only fresh, good quality skimmilk should be used and all steps in the manufacturing process must be scientifically controlled. Cottage cheese must be packaged in a room free from contamination by airborne organisms. The cultures must be pure and they must be handled properly. Temperatures must be controlled throughout processing and storing, as well as through all channels to the final consumer. The water supply must be free from spoilage organisms.

CHANCES IN PROCESSING AND PRESENT BLAND FLAVOR OF COTTAGE CHEESE

It is the bland, mild acid, and clean flavored cottage cheese that has increased consumption in western and midwestern states. This type of cheese has resulted from carefully controlled methods, use of coagulating agents to cut the curd and cook at lower acidities (higher pH), and the trend towards making small curd, which makes it easier to remove the whey and to obtain mild-flavored cottage cheese. Some years ago, consumers of cottage cheese and other dairy products expected strong flavors. Cottage cheese was frequently sour, high acid and, at times, had fermented and yeasty flavors.

Research, improved equipment, modern controlled methods, good refrigeration, and better handling made it possible to produce fairly uniform and mild-flavored cottage cheese. The consumer quickly notices any feed, absorbed, chemically produced, or bacterial off flavors, and consumption decreases immediately if these defects are not corrected. One must also consider that the time between actual processing and the time cheese reaches the consumer has increased and that there is very little hope that this condition can be changed in the future.

We must be prepared to produce a dry or creamed cottage cheese that will retain a fresh, clean, mild acid flavor for 7 to 14 days. The following factors will be discussed in some detail.

1. Present methods and practices of cooking cottage cheese curd.
2. Suitable processing and packaging area or rooms for cottage cheese manufacturing.
3. Water supply, pH control, and chlorination.
4. Preparing and handling of cream dressing.
5. Temperature control throughout processing, storage, and delivery.
6. Laboratory control measures.

PRESENT METHODS AND PRACTICES OF COOKING COTTAGE CHEESE CURD

In the days of flake or cube cottage cheese manufacture, where coagulating agents and raw skim were used, operators tried to avoid firm curd. It was the usual practice to finish cooking at 106 to 114° F., with very little or no holding in the whey. Recently, the majority of manufacturers cool the curd in water at 38 to 45° F. The use of lower cooking temperatures left large numbers of starter organisms in the curd which was put into the refrigerator at 60 to 75° F. There was no psychrophilic, slimy, gelatinous type of spoilage that is so common today, as these organisms were inhibited by the lactic acid-producing organisms. The common spoilage then was caused by yeasts, molds, and coliform bacteria.

Pasteurization of skim for cheese became compulsory and required higher cooking temperatures. With better equipment and sanitary practices, and cooling the curd in the vat, more attention was given to air contamination, separate processing rooms, refrigeration, and treatment of water supplies. These changes have affected the microbiological flora of finished cottage cheese curd and creamed cottage cheese, so that we now have more spoilage of the gelatinous, slimy, putrid, fruity, and fermented types.

The data in Tables 2 and 3 express the importance of pure water supplies, sanitation, prevention of contamination, and low temperatures after the whey is removed and the curd is processed and finally packaged. Most microorganisms (Table 1) are easily killed at legal pasteurization temperatures. Although most of these organisms, even the Pseudomonas group which are classed as psychrophiles survived 120° F. for 15 min. in skim at pH 6.7, only one of 17 of these cottage cheese spoilage organisms survived the same heat treatment in whey at pH 4.55. The mold, *Geotrichum candidum,* which survived two out of three trials, might possibly be expected to survive, as molds are more acid-tolerant.

Because most cottage cheese manufacturers cook the curd to 120° F. or higher, and hold it in the whey at a pH of 4.55 to 4.68 for 15 min. or longer, we must assume that the curd at the time of draining the whey is relatively low in microorganisms. The curd must not be

TABLE 1

Ability of organisms that spoil cottage cheese to grow at 120° F. in skimmilk and their inability to grow in whey at pH 4.55 (1)

Microorganisms	Growth in skimmilk	Growth in whey
1. *Achromobacter butyri*	+	−
2. *Achromobacter eurydice*	+	−
3. *Alcaligenes metalcaligenes*	+	−
4. *Escherichia coli*	+	−
5. *Escherichia freundii*	+	−
6. *Micrococcus candidus*	+	−
7. *Micrococcus conglomeratus*	−	−
8. *Micrococcus flavus*	+	−
9. *Pseudomonas desmolyticum*	−	−
10. *Pseudomonas fluorescens*	+	−
11. *Pseudomonas fragi*	+	−
12. *Pseudomonas tralucida*	+	−
13. *Rhotorula flava*	+	−
14. *Torula candida*	+	−
15. *Geotrichum candidum*	+	+*
16. *Mucor plumbeus*	+	−
17. *Penicillium frequentans*	+*	−

+—growth; −—no growth; *—growth in two of three trials.

Numbers 1 to 12 are bacteria, 13 to 14 yeast, and 15 to 17 molds.

TABLE 2

Bacteria counts from samples of pasteurized milk exposed in processing areas

Samples exposed	Coliform	Standard plate count	Psychrophilic count
		(No. per ml.)	
In cheese refrigerator	Less than 1	4	25
On cheese filling machine	Less than 1	11	100 T
Starter processor	Less than 1	15 + mold	TNTC [a]
On cheese dressing vat	Less than 1	6	TNTC
In empty cheese vat No. 2	Less than 1	3	TNTC
Cream dressing	1	1.3 T	110 T
City water, pH 9.3	Less than 1	200	2.3 M

[a] Too numerous to count with the dilutions made.

TABLE 3
Bacteriological analyses made on water supplies

Dairy location	Standard plate count At 32° C.	Standard plate count 5 days at 7.2° C.	pH	Remarks
	(No. per ml.)			
Illinois	4M[a]	120M	7.5	Water from well which was being chlorinated
Arkansas	14M	11T	7.7	First sample
Arkansas	380T[b]	300	7.8	Same plant 2 wk. later
Texas	18M	14M	8.1	Plant had trouble with psychrophilic spoilage
Connecticut	28T	100	7.1	
Iowa	250T	47T	9.1	
Nebraska	390T	200	8.0	
Texas	70	20	8.3	
Kentucky	5T	63T	8.0	
Texas	84T	880	9.6	
Alabama	TNTC[c]	39T	8.1	
St. Louis, Mo.	200	TNTC	9.3	
Oklahoma	14	39T	10.1	
Michigan	15M	220T	7.7	Before treatment
Michigan	2	Less than 10	.....	After treatment
St. Louis, Mo.	100	10	9.5	Before treatment
St. Louis, Mo.	1	Less than 10	6.1	After treatment
Ohio	78T	150M	8.0	Before treatment
Ohio	Less than 1	210	6.4	After treatment

Note: Sterile nonfat milk was added to water samples before plate counts were made and held at 72° C.

[a] Millions.

[b] Thousands.

[c] Too numerous to count for the dilution used.

contaminated from this point by water, air, cream dressing, unclean utensils, and general unsanitary practices.

SANITARY PROCESSING AND PACKAGING ROOMS

Air contains bacteria and it is important that the processing and packaging rooms be constructed of hard-finish, smooth, washable walls, have well-drained, smooth floors, and ceilings that are smooth and free of fixtures and pipes, especially above vats and packaging equipment. Openings or doors should be held to a minimum, filtered air under pressure should enter processing and packaging rooms, and filters should be removed when they become contaminated. Filtered and refrigerated air should be provided for packaging.

The data in Table 2 were obtained in a Midwestern plant which produced uniform cottage cheese of good flavor consistently, using modern, controlled methods. The operator had consistently tried to get a shelf-life of from five to seven days; whereas, a shelf-life of from seven to 14 days at 40 to 45° F. should be achievable. The manufacturing and packaging areas were undesirable. During a three- to five-day period, ¼ in. of pasteurized skimmilk was placed in sterile Petri dishes and exposed around the processing area for 20 min. The SPC was made and plates were incubated 48 hr. at 32° C. Samples were held at 45° F. for six days and a second SPC made at 32° C., to observe for any (Moseley Method) psychrophilic growth.

This is a case of a very poor processing area which contributed to a shortened shelf-life of well-made cottage cheese. This factor must be considered by all cheese manufacturers.

CONTROL OF PH AND CHLORINATION OF WATER SUPPLY

A great deal of research has been done since the work by Parker *et al.* (3) was reported. There should be no question that the water supply is a most important factor in keeping quality. It has been well established that water is a source of organisms of the psychrophilic group and they have been isolated from cottage cheese which has spoiled on the shelf.

Hundreds of samples of water from cottage cheese plants all over the United States have been gathered by this writer and sent to the Moseley Laboratory in Indianapolis for bacteriological analyses. The analyses of water supplies from plants for 1954 to 1958 are shown in Table 3. It is evident that the original SPC was rather high in many cases and this was due to length of time in transit, as the samples were not refrigerated.

It is clearly shown in Table 3 that water can be the cause of poor keeping quality, especially for the psychrophilic type of spoilage. The pH of water was found to vary from 7.0 to 10.2 in plants where these samples were obtained.

Cottage cheese wash-water at pH 8.5 to 10.0 sometimes causes slick or slimy appearance due to the formation of sodium caseinate. The higher pH range also favors the growth of many psychrophilic organisms. For this reason, all water should be filtered, then phosphoric or citric acid added to lower pH to 5.5 to 6.0, then 5 to 8 p.p.m. chlorine added and held 60 sec. or more before cooling the curd in the vat. Water treated in this way will be nearly sterile. It is important to acidify first, as chlorine is more effective at lower pH values.

TEMPERATURE CONTROL THROUGHOUT PROCESSING, PACKAGING, STORING, AND DELIVERING OF COTTAGE CHEESE

It is of great importance to control temperatures throughout processing, packaging, storing, and delivering of cottage cheese. The curd temperature in the last wash water should be 40° F., the cream dressing 40° F. or below, and the temperature during creaming and packaging should not increase more than 5° F. Storage temperatures should be below 40° F.

During 1955 through 1958, many lots of cheese were made in the Meyer-Blanke Laboratory and observed for keeping quality, studying temperature of storage, preparation of dressing, dressing pH, water with or without acidification, chlorination, cooking temperatures, etc. All curd was made by short method and it was washed three times at 40 to 45° F., creamed after draining 45 min., then placed in chlorinated plastic packages. Split samples were stored at 47 and 38° F. The lots, good or poor, some purposely contaminated, kept twice as long at 38 as at 47° F. Low temperatures contributed to good keeping-quality.

A shelf-life for cottage cheese at 38° F., from seven to 83 days, was obtained and many lots kept for 24 to 63 days. Samples with a shelf-life of 30 days or more had low bacterial counts at both the second and seventh day after being made and packaged, except one lot, where starter was added to dressing, and another where 0.04% by volume sorbic acid was added. All samples with over 17 days of shelf-life were free of coliforms, yeasts, and molds.

PREPARING AND HANDLING CREAM DRESSING

We believe from many years of research and practical work with manufacturers that the quality and special processing and handling of dressing is probably the most important single factor that affects shelf-life. Many cases of cottage cheese spoilage are due entirely to the poor quality of cream dressing.

The butterfat content of cottage cheese dressing will vary a great deal according to method of manufacture, size of curd, method of creaming and mixing (hand or mechanical), firmness of curd, air drying, etc. It will usually vary from 11 to 17% fat, and in many cases 1 to 2% extra nonfat solids added with salt to give a salt content of 0.90 to 1.10% in the final product. Some add a small amount, 1 to 2%, of culture to dressing before heating, in an attempt to reduce pH, as the pH of cream will be 6.6 to 6.7. A ratio of 1 lb. of 12% dressing (pH 6.7) to 2 lb. curd (pH 4.68) will make a pH of 5.1 to 5.2 in the final creamed cheese. We have used a blend of milk solids, powdered organic acids, and stabilizer to reduce the cream dressing pH from 6.6 to 6.0, and after heating to 165° F. and holding 30 or 45 min. and cooling with or without homogenization, this dressing had a very low bacterial count. We believe that a pH of 4.95 to 5.05 on final creamed cottage cheese, other factors being controlled, does improve the keeping quality.

The addition of the proper amount of starter distillate can improve the flavor of cottage cheese. We have recommended 1 to 5% of a good lactic culture in the chilled dressing just before adding it to the curd, and many operators still use this method. It adds flavor and helps to retard the growth of psychrophilic organisms and the gelatinous type of spoilage, but is detrimental to quality if temperatures are not kept low, as millions of organisms are added to the cheese.

Sorbic acid in cheese dressing amounting to levels of 0.03 to 0.05% in final creamed cottage cheese has been investigated by several workers. It is primarily used to retard yeasts and molds, but also has been found effective against other spoilage organisms. The use of sorbic acid is not legal and possibly it should be considered in the future, not to mask poor quality but to further extend the shelf-life of good quality creamed cottage cheese.

The many thousands of bacterial counts made by the Moseley Laboratory for their regular commercial customers, who sent samples of cottage cheese, dry or creamed, cheese dressing, and cream of varying fat contents during the past 5 yr., do not show that all low-count cheese has a good shelf-life and that all high-count cheese has poor keeping-quality. The type of predominating organism is the important factor.

The second SPC (standard plate count 48 hr. at 32° C.) after five days of storage at 45° does have a relationship to shelf-life. Moseley (2) reports that high initial counts for fresh cottage cheese are usually due to high thermoduric counts in the skimmilk, addition of starter to dressing, or low cooking temperatures.

LABORATORY CONTROL MEASURES

The data presented indicate the need for good laboratory controls as an important factor in maintaining a long shelf-life for cottage cheese. Bacteriological tests can be used to assure quality milk supply for cottage cheese, check and control contamination, help to ascertain the suitability of wash water, and make three- to five-day holding tests at 72 and 45° F. The longer five-day holding of samples at 45 to 50° F. is preferred to the shorter time at 72° F.

TABLE 4

Bacterial conts on dressing for cottage cheese as reported by the Moseley Laboratory [a] from 1955 to 1958

Source	Fresh	Coliforms	Yeast molds	Stored at 45° F. for 5 days	pH	Shelf-life	Remarks
	(SPC/g)					(days)	
Meyer-Blanke Lab. 1	−3T[b]	0	0	−3T[e]	5.0	83	Stored at 38° F. Stale
Meyer-Blanke Lab. 2	460T	0	0	280T	4.9	10	Low-temp. cook, sour, high-acid
Meyer-Blanke Lab. 3	315T	0	0	220T	5.1	31	Culture added to dressing
Meyer-Blanke Lab. 4	10T	0	0	2T	5.0	60	NU-CD[d] dressing plus sorbic
Meyer-Blanke Lab. 5	6T	0	0	25T	5.0	46	Bitter
Meyer-Blanke Lab. 6	12T	0	0	4T	5.0	51	18% dressing plus NU-CD[d] heated
Meyer-Blanke Lab. 7	12T	0	0	139T	5.2	23	18% dressing (same cream and salt without NU-CD)
Plant P dry curd	390T	−10[e]	−10[e]	260T	....	....	Plant had short shelf-life
Plant P cream dressing	73M[c]	73T	− 1[e]	170M	....	....	Dressed at this plant
Plant P creamed cheese	1M	11T	−10[e]	97M	..	....	Finished cheese at this plant
Plant 5 creamed cheese	−3T	− 1[e]	− 1[e]	140M	5.3	....	Psychrophilic spoilage
Commercial dressing	77T	....	....	12M	6.7	....	Coffee cream used
Creamed cheese	29T	0	0	25T	5.0	27	Processed dressing plus NU-CD[d]
Plant 8 creamed cheese	20M	0	0	13M	....	....	Pasteurized skim had SPC of less than 3T
Plant 4 creamed cheese	−3T	0	0	−3T[e]	....	....	Pasteurized skim had SPC of less than 3T
Plant 1 dry curd	230T	0	0	400T	4.7	10	Well-operated plant
Plant 4 creamed cheese	215T	17T	5	28M	5.2	....	Plant poorly operated
Plant 4 cream dressing	38T	11T	0	49M	6.7	....	Same plant
Plant 4 city water	200	0	0	TNTC	8.9	....	Same plant

[a] Indianapolis, Indiana.
[b] Thousands.
[c] Millions.
[d] Mixture of milk solids, organic acids, and stabilizer.
[e] Less than number indicated.

Production and consumption of cottage cheese have increased more than those of any other dairy product in recent years and, to maintain this trend, it is necessary to maintain uniformity of quality and greater keeping quality.

The improvements of equipment, better scientific knowledge of controlled processing, and improved sanitation have resulted in a mild-acid, clean-flavored cottage cheese that the consumer demands. This is true of other dairy products and the consumer is quick to notice any off-flavor; therefore, we must maintain greater uniformity of a quality for a longer period.

Improved shelf-life must begin with a good raw product, then be followed step by step through proper pasteurization, culture control, careful processing, good sanitary practices, treatment of water supplies, and control of temperatures.

Many cottage cheese spoilage microorganisms, in fact, most of them, do not survive cooking temperatures of 120° F. in the whey for 15 min. at pH 4.5–4.7.

We must assume that postcooking contamination is a real problem, and that the water should be controlled to a uniform pH and made as sterile as possible, dressing carefully, processing with pH control, and that creaming, packaging, temperatures, storage, and delivery must be closely controlled to avoid contamination.

Data on bacterial counts of creamed cottage cheese do not give a definite correlation with low counts, but a great deal can be learned by control of procurement, processing, and handling to obtain better keeping quality.

The possibility of future use of sorbic acid in dressing may be useful in extending shelf-life. The proper care and use of a good culture in dressing may help to retard psychrophilic spoilage, but it must be kept at a low temperature.

Cottage cheese making is not simple. It is very complex and needs to be understood, with careful control to maintain quality and a satisfactory shelf-life of 14 days at 40 to 45° F.

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OUR ASSOCIATION

REPORT OF THE JOURNAL MANAGEMENT COMMITTEE FOR 1959

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The Journal Management Committee for 1958-59 was most fortunate in succeeding to the fine work by W. M. Roberts and his committee.

We began the new year with the editing and publishing problems well stabilized and with a new plan for abstracts. The elimination of the abstract section from the JOURNAL and the formulation of the membership subscription plan for *Dairy Science Abstracts* was accomplished with the help of H. F. Judkins, our energetic Secretary. It was not necessary for the Association to subsidize this new plan, because sufficient members subscribed for this new abstract service.

During the fall of 1958, considerable study was given to the printing and mailing date of the JOURNAL. This resulted in a plan to strive for a mid-month mailing date. This would insure receipt of the JOURNAL within the month that it was printed. This goal has been attained.

Book reviews, which were formerly carried in the abstract section of the JOURNAL, are being continued in a special section under a separate title, as of January, 1959.

The People and Events section of the JOURNAL had been organized, monthly, by R. J. Dandeneau, who was supervisor of our publication at the Garrard Press. He resigned in August, 1958, to accept a professorship at Southern Illinois University. Arrangements were approved by our committee to have Mr. Dandeneau continue this work on the same basis as when he was employed by the Garrard Press.

With the termination of the abstract section of the JOURNAL, which had been handled by W. O. Nelson, it appeared feasible to make new arrangements for the annual index which had been prepared under his direction. The Editor-in-Chief's office will assume the duties of indexing and this change was approved by the committee.

The work of the Editorial Board and that of the Editor-in-Chief was reviewed and the committee commended them for the quality of their work.

JOINT MEETING OF THE JOURNAL MANAGEMENT COMMITTEE AND EDITORIAL BOARD AT THE ANNUAL MEETING

D. H. JACOBSEN, Chairman

The Journal Management Committee met in joint session with the Editorial Board on June 16, 1959. The agenda for discussion had been presented to all members of these groups by letter on May 25, and the lively discussion in the meeting indicated that each member had given some thought to the problems outlined.

Ideas for review papers were solicited and the members provided enough working ideas to insure the continuous production of this feature in the JOURNAL. Additional areas for review papers were suggested and the members of the group were requested to suggest authors who could be invited to prepare the reviews.

The question was raised, Should we accept technical notes of research where negative results were obtained? The discussion which followed resulted in agreement that such contributions should be judged on the basis of the scientific value of the report. The project design and methods developed might well be worthy of publication as a scientific contribution.

Further discussion on the establishment of a criterion of quality on papers for the Our Industry Today section indicated general approval of the material now being published. It was the general conclusion that we should rely on the editor's judgment rather than to attempt any kind of fixed standard, and that major decisions such as comprehensive reports of sectional meetings be referred to the Journal Management Committee.

The time between submittal of papers and final approval for publication has been extended in some cases by long delays in the channels of review. Suggestions of fixed dead-

lines for reviewers were discussed. It was quite apparent, however, that this method would not be very popular with our unpaid Editorial Board Members. They called attention to the rather complex nature of the editorial job for some papers which required study and revision. It would appear that such problems can be solved only by careful selection of the individuals on the Editorial Board and with assurance that they are interested in serving as reviewers. Secondary referrals of papers to specialists is a practice in some fields of research. The only way to overcome this situation would be through the enlargement of the Editorial Board, which would not be feasible at this time.

The question of, How much editing should be expected on papers? was raised by members of the Editorial Board. Some manuscripts are so poorly written that extensive rewriting is obviously necessary. It was agreed that such manuscripts should be returned to the authors for correction and with the suggestion that editorial review within the college or university department become a regular practice. Since the majority of manuscripts are of fair quality, and the poorly written manuscripts come from relatively few institutions, it is suggested that more attention be given to this problem by those who have been involved with such papers.

Improvement in quality of papers should be a continuous objective of the Association. This improvement can be achieved if department heads will set and vigorously maintain high standards of scientific performance in their research activities.

The limitation on length of papers has caused some authors to present a research report in a series of short papers. While no action was taken on this problem, it was the general opinion of the joint meeting that the justification for a longer paper, rather than a series, should be passed on by the Editor-in-Chief and the Editorial Board, based on the merits of the paper in question.

THE EDITOR-IN-CHIEF'S REPORT FOR THE 1959 ANNUAL MEETING

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During 1958, 271 papers were published in the five sections of the JOURNAL OF DAIRY SCIENCE. Six reviews were published and, judging by orders and requests for reprints, they were received with much interest by the membership and by investigators in related fields. Reviews on subjects of timely interest are being written for the JOURNAL by authorities in their respective fields. Table 1 contains a detailed summary of all papers published in the JOURNAL for 1958. The general summary is as follows:

	No. of papers
Production	146
Manufactures	105
Production-Manufactures	20
Total	271

The JOURNAL is growing. Volume 41 of the JOURNAL for 1958 contained 1,844 pages, as compared to 1,666 pages in Volume 40 for 1957. Volume 42 for 1959, including the May issue, contained 944 pages as compared to 751 pages for the same period in 1958.

A study was made of the number of papers published in the JOURNAL during its lifetime. From 1917 to 1946, the number of papers in Manufactures exceeded those in Production, but from 1947 to the present time, more Production than Manufactures papers were published. The results are shown in Figure 1. The increased numbers of papers in the general area of production since 1947 have been due to accelerated research activities in artificial insemination, breeding, nutrition, and physiology.

The quality of papers submitted for publication in the JOURNAL can be improved. The principal reasons for the delays in publishing some papers were as follows:

1. Experiments were not designed correctly.
2. Results were not statistically analyzed in order to provide a basis for the correct interpretations of data.
3. Papers were not written clearly and concisely.
4. Papers did not conform to the JOURNAL style.

There is not much we can do about experimental designs, except to emphasize their importance. We can, however, insist on more precise compliance with Points 2, 3, and 4.

The abstracts submitted by authors for the regional and annual meetings of the American Dairy Science Association can be greatly improved. Many abstracts submitted for the regional meetings needed to be revised and retyped in the editor's office because there was not enough time to return them to the authors. A number of abstracts submitted for the annual meetings this year were either revised and retyped by the chairmen of the different sections or were returned to the authors for further revision.

The chairmen of the program committees for the Manufacturing, Production, and Extension

TABLE 1

Distribution of papers published in Volume 41 of the Journal of Dairy Science for 1958

Month	Research Papers M[1]	Research Papers P[2]	Our Industry Today M	Our Industry Today P	Technical Notes M	Technical Notes P	Review Papers Symposium M-P[3]	Symposium M	Symposium P	Our Association M	Our Association P	Our Association M-P
	(No. in each group)											
January	10	14	1		1	2				1		4
February	3	9	1		4	2		4				1
March	7	3	2	1		1		1	3	1		
April	6	5	3		1	1						1
May	5	9	1			3	1					1
June	5	8		2	1	1						1
July	7	6			4	3	1					
August	2	13	3			3	1					1
September	7	7	1		3	2	1			1		
October	3	13		3		1	1			1		3
November	5	14		1		2						
December	5	13	2			1	1			3		2
Totals	65	114	14	7	14	22	6	5	3	7	0	14
Grand Totals	179		21		36		6	8		21		

[1] Manufactures.
[2] Production.
[3] Apply to both Manufactures and Production.

Sections did a thorough job this year of transmitting to the Program Committee at the University of Illinois abstracts that were well written and edited for JOURNAL style. It was a pleasure to submit them to the Garrard Press.

Robert Garrard and his staff at the Garrard Press have been very cooperative in publishing the JOURNAL and have met contingencies beyond the call of duty. Charles Hopper assumed the duties of R. J. Dandeneau. His interest in the JOURNAL, and especially his efforts to achieve an earlier monthly publication date, is appreciated by the membership in the Association and by the editorial staff. Mrs. Lois Hough has also been of much assistance to the editorial staff.

It is time to consider the publication of a new Guide for Authors. The Conference of Biological Editors has compiled some useful information which should be published during late 1959 and from which we should be able to prepare a useful set of instructions for our authors.

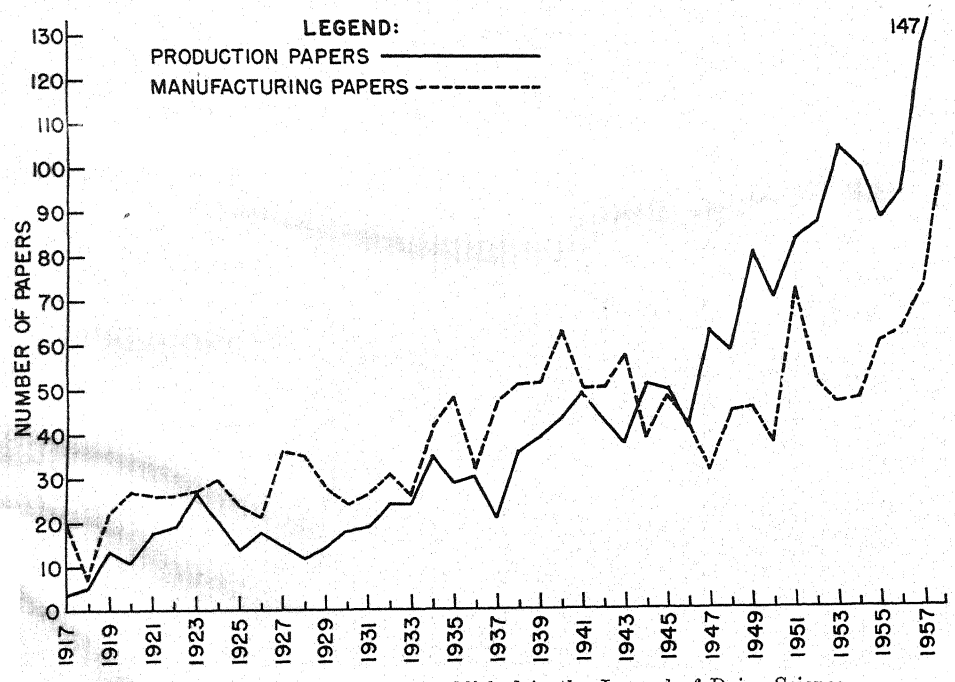


FIG. 1. Distribution of papers published in the Journal of Dairy Science

BOOK REVIEW

THE TECHNOLOGY OF FOOD PRESERVATION. Norman W. Desrosier. The Avi Publishing Company, Inc., Westport, Connecticut. 418 pp. 1959.

The purpose of this book is to present the elements of food preservation technology. It is ideally suited as a text for introductory food technology courses. Since this book covers the major phases of the food industry in a comprehensive and authoritative manner, it should be an ideal reference for all those who are interested in food technology.

The author has refrained from the consideration of techniques for the processing of specific products and this has allowed a good coverage of the fundamental science involved in a number of processing and manufacturing areas.

The diverse material of this book is presented in 11 chapters. An abundance of figures, pictures, and tables has been used by the author and this greatly enhances the reading quality of the book. The first three chapters are devoted to introduction, history, and the general problems involved in feeding mankind. Separate chapters are devoted to the preservation of food by canning, freezing, and drying. Although all the chapters are quite adequate, the one on canning was considered best, with freezing and drying second and third, respectively.

The chapter on fermentation and pickling was very well done and is highly recommended. The food as sugar concentrates chapter is perhaps the least desirable offering in the book. Although the material presented in this chapter is good, the criticism lies in the lack of complete coverage of food preservation in this area. The chapters on chemical additives and ionizing radiations are excellent. The comment might be offered that many food technologists may not share the author's enthusiasm for the extensive use of ionizing radiations in the food industry. However, this does not, in any way, detract from the general value of this chapter.

The appendix of this book is devoted to a number of very useful tables, as well as a complete list of journals which cover the many aspects of food preservation. The author should be complimented on the inclusion of so much information in a single volume of 418 pages. This book is a significant addition to the food technology library.

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